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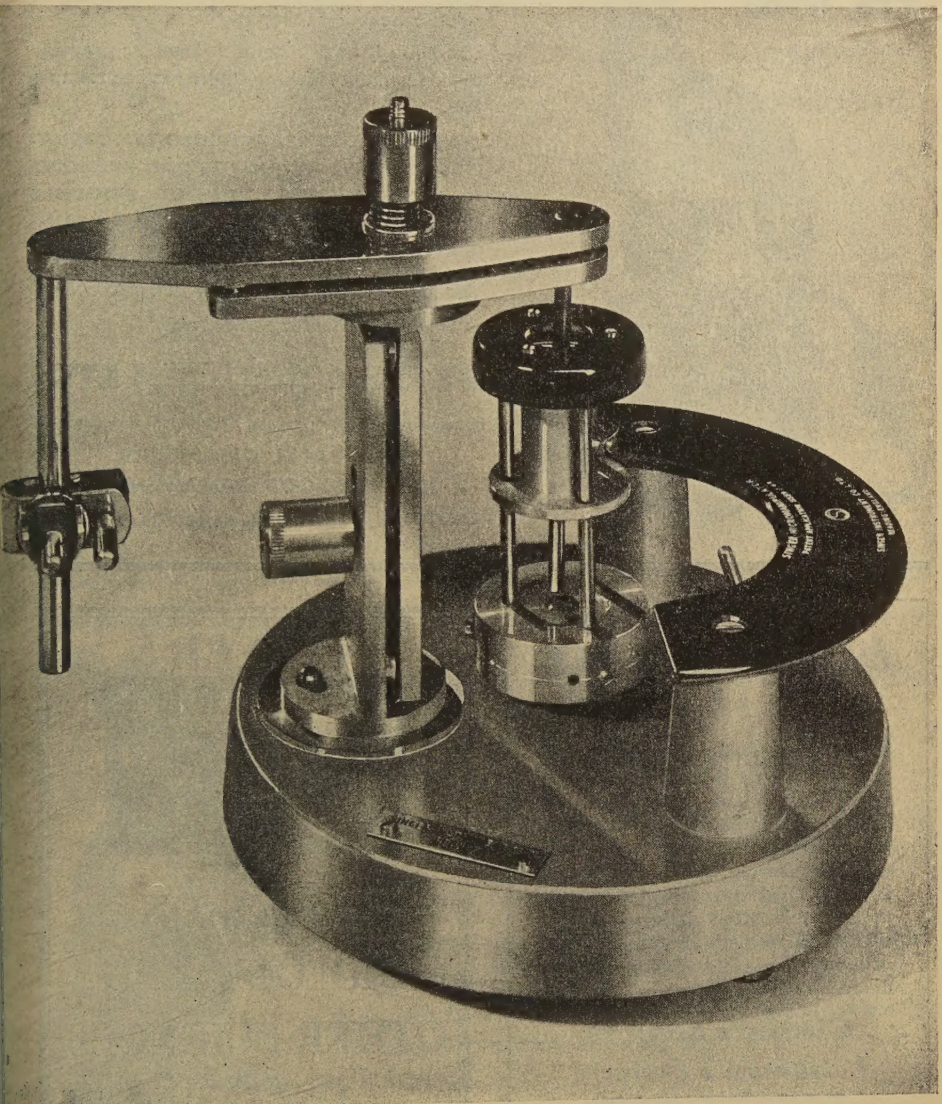

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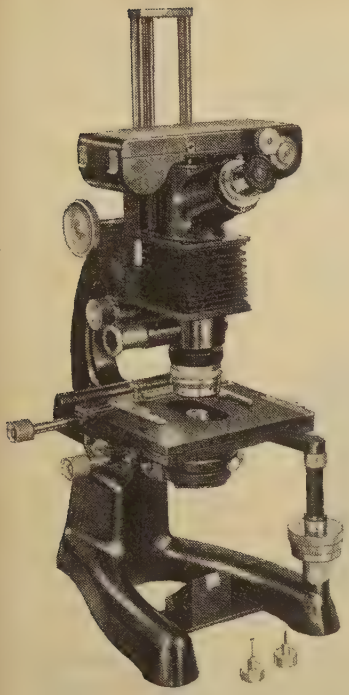

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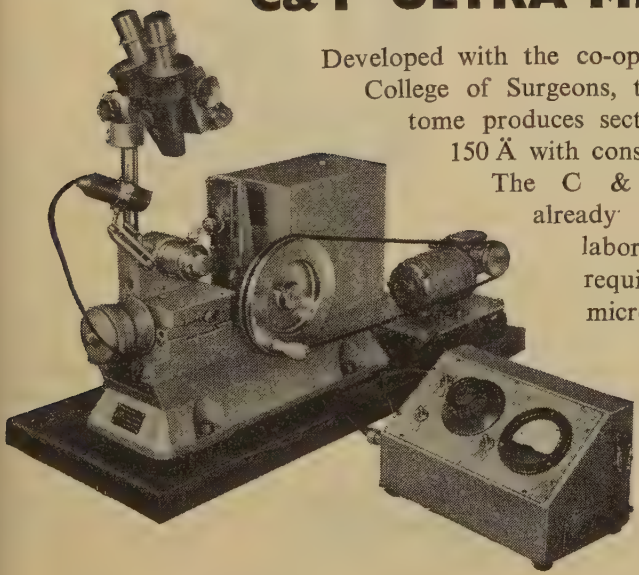

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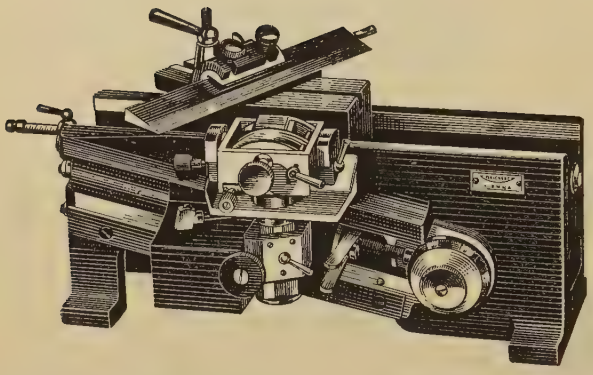

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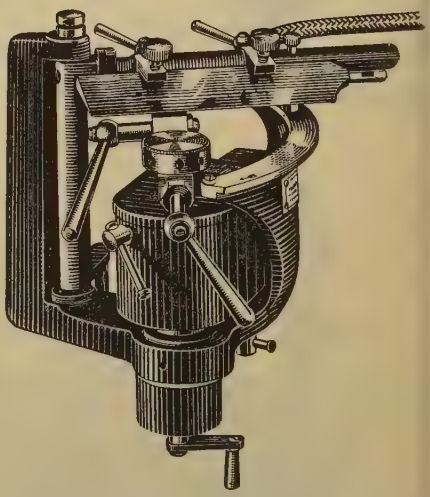

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Formation and Involution of Striated Muscle Fibres during the Growth and Moulting Cycles of *Rhodnius prolixus* (Hemiptera)

By V. B. WIGGLESWORTH

(*From the Department of Zoology, University of Cambridge*)

With two plates (figs. 1 and 2)

Summary

The ventral intersegmental muscles of the abdomen in *Rhodnius* undergo a cycle of development and involution during each larval stage. They are fully developed only at the time of moulting or hatching from the egg. Within 3 or 4 days after moulting the fibrils have disappeared; but the nuclei, with a little cytoplasm containing mitochondria, survive within the highly folded muscle-sheath.

The formation of fibrils begins between 2 and 3 days after feeding. At first they are uniformly birefringent. The striations appear later, and the muscles will then contract after transection. The fibrils are 0·1–0·2 μ thick when first formed; they grow by intussusception and splitting to a thickness of about 0·8 μ.

Succinoxidase first appears in quantity in the mitochondria at the time when striation and contractility develop. It disappears within 3 days after moulting, during the involution of the fibrils. The distribution of ribonucleic acid in the developing muscle is described.

Involution results from autolysis which begins around the nuclei in the centre of the muscle. The phagocytic blood-cells play no part in the break-down.

The rich nerve-supply to the muscles persists apparently unchanged throughout the cycle of involution; and the cycle of growth occurs normally after section of the nerves.

THE blood-sucking bug *Rhodnius prolixus* Stål takes only a single meal of blood in each of its five larval stages. These large meals may exceed twelve times the weight of the unfed insect and cause enormous distension of the abdomen. In order to allow for this stretching of the body-wall, the cuticle of the abdomen is highly extensible (Wigglesworth, 1933), and at the time of feeding, as will be shown in this paper, the intersegmental muscles are wanting.

The intersegmental muscles of the abdomen play an essential role during moulting in insects: by their contraction they raise the hydrostatic pressure of the blood and so bring about the expansion of the newly formed appendages. It has been found that in *Rhodnius* these muscles are developed only in preparation for the act of moulting; after moulting they rapidly break down.

This repeated cycle of muscle formation and involution, which does not appear to have been described in other insects, affords favourable material for observing some of the histological details in the development and break-down of muscle-fibrils.

Material and Methods

All five larval stages of *Rhodnius* have been used, but most observations have been made on the 1st and the 4th stages. When kept at 25° C the interval between feeding and moulting is 11 days in the 1st stage and 14 days in the

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4th stage. After moulting these insects will survive several months without feeding again.

The tergites and sternites are isolated by cutting along the margins of the abdomen and removing the integument with the adherent epidermis, fat-body, and muscles. For studying in polarized light these isolated parts are fixed in neutral formol and the fat removed with xylene after dehydration in alcohol; they are then returned to water and mounted in Farrants's medium. For close study the muscles are scraped away from the cuticle with a dissecting needle and mounted in the same way.

Whole mounts of the sternites, &c., have been fixed in Carnoy's or Bouin's fixative and stained withHansen's trioxyhaematein, the muscles being isolated as above for closer study. Mitochondria can be studied in similar whole mounts, after fixation in 1% osmium tetroxide in phosphate-citrate buffer at pH 6·8, if stained with Hansen's trioxyhaematein and mounted in Farrants's medium. Tangential and transverse sections of the sternites, after double embedding by Peterfi's method, have been stained with Masson's trichrome stain (modification of Foot) and other stains.

Ribonucleic acid has been followed by the use of pyronin/methyl green, and with gallocyanin at pH 1·6 (Lagerstedt, 1947); controls (both whole mounts and sections) being treated for 2 hours at 37° C with 0·1% ribonuclease (Armour) previously heated at 90° C for 10 minutes to inactivate protease and deoxyribonuclease.

The development of cytochrome oxidase and succinoxidase has been followed by the Nadi reaction and the neotetrazolium method of Shelton and Schneider (1952).

Nerve-endings have been stained with methylene blue injected into the intact animal.

Cycles of Growth in the Intersegmental Muscles

The intersegmental muscles consist of strap-like bands running from the anterior margin of one segment to the anterior margin of the next. On the tergites (for example, in the 5th-stage larva at the time of moulting) the muscles are more or less vestigial. They are moderately developed on segment 1, vestigial on segments 2–6, slightly more developed on segment 7, and again fairly well developed on segment 8. Fig. 1, A shows these slender striated

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Fig. 1 (plate). A, part of abdominal tergites of newly moulted 5th-stage larva of *Rhodnius*, showing the slender muscles on segments 6 and 7, with the dorsal heart in the mid-line. Polarized light.

B, sternites of abdomen of newly hatched larva. Polarized light.

C, the same, 3 days after hatching.

D, sternal muscles in 1st-stage larva 4 days after feeding. Polarized light.

E, the same at 5 days after feeding; striation appearing.

F, the same at 10 days after feeding; muscles fully formed.

G, H, sternal muscles in 4th-stage larva at 4 days after feeding. Polarized light.

J, the same at 7 days after feeding.

K, the same at 11 days after feeding.

L, the same in newly moulted 5th-stage larva.

FIG. 1

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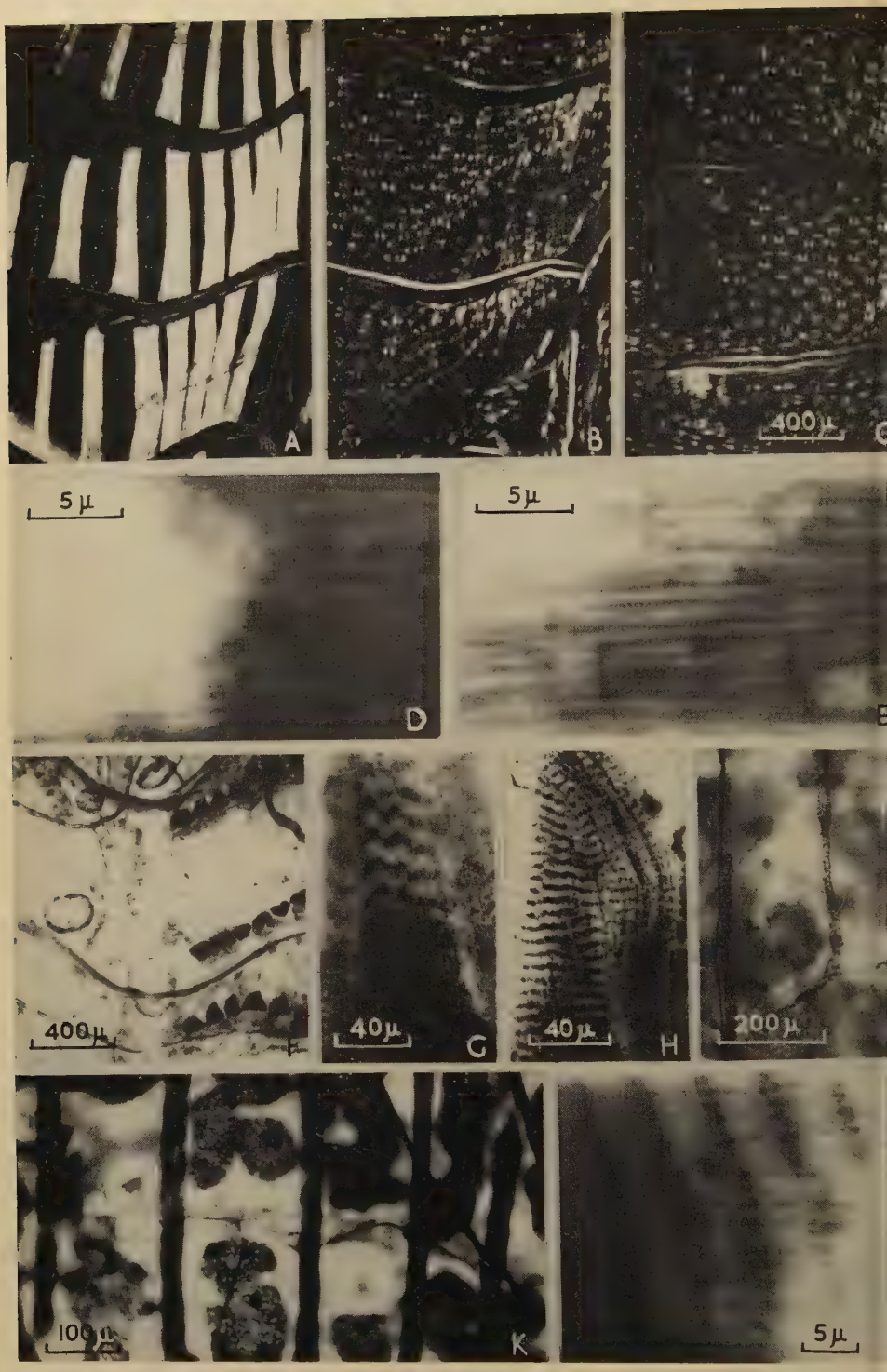

FIG. 2

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muscles on segments 6 and 7. All disappear completely within 4 days after moulting. (In fig. 1, A, and other similar figures, the small 'Maltese crosses' which form the background are produced by the dome-shaped plaques scattered over the surface of the cuticle—the cuticle being birefringent when viewed in profile.)

The muscles of the sternites are much better developed, and only these have been studied in detail. There are nine muscle-bands on each side of each segment. (As a rare anomaly a muscle-band may extend across two segments without intermediate insertion.) When the *Rhodnius* larva hatches from the egg the sternal muscles form almost a continuous sheet (fig. 1, B); by 3 days after hatching muscle-fibres have completely disappeared (fig. 1, C). When the 1st-stage larva is fed, birefringent muscle-fibrils begin to appear within 3–4 days (fig. 1, D); they are becoming more conspicuous and striations are appearing by 5 days (fig. 1, E); and they are fully developed by 10 days (fig. 1, F). At this and later stages the muscles show gaps between them and do not form a continuous sheet as in the newly hatched insect. The 1st-stage larva moults to the 2nd stage at 11 days after feeding and the muscle-fibres disappear again within 3 or 4 days.

A similar cycle occurs after feeding in each stage. Fig. 1, G–L shows four stages in the development of the muscles when the 4th-stage larva moults to the 5th stage. Fig. 2, A shows the muscles of the 5th stage at the day of moulting. In fig. 2, B, taken 3 days later, the muscles can just be seen as 'ghosts' running across the background of 'Maltese crosses'. In fig. 2, C, at 5 days after moulting, the muscles have completely disappeared.

The nerve-supply to the sternal muscles is conspicuous. The nerves, which are formed by branches from the fused ganglionic mass in the thorax, run transversely across the middle of the bands giving off numerous branches to them (figs. 2, K; 9, p. 478).

State of the Muscle in the Resting Phase

Although the conspicuously birefringent muscle-fibrils disappear soon after moulting, the muscle-sheath and the nuclei remain. In a recent paper (Wig-

Fig. 2 (plate). A, sternites of abdomen in newly moulted 5th-stage larva. Polarized light.

B, the same at 3 days after moulting. Muscles are just perceptible as 'ghosts', particularly in the middle segment.

C, the same at 5 days after moulting. Muscles no longer show birefringence.

D, horizontal section of muscle of 4th-stage larva at 3 days after feeding. Fibrils 0·1–0·2 μ thick are present. Masson's trichrome stain.

E, the same at 7 days after feeding. Fibrils now 0·3–0·4 μ thick.

F, abdominal sternites of 4th-stage larva 12 days after feeding. Neotetrazolium test for succinoxidase. Strongly positive in the contracted muscle stumps on the right; negative in the uncut muscles on the left.

G, sternal muscle from newly moulted 5th-stage larva. Neotetrazolium test for succinoxidase strongest in the I bands (compare fig. 5, D).

H, thoracic muscle similarly treated.

J, nerves to the abdomen showing positive neotetrazolium test in the sheaths.

K, sternal muscles in abdomen of newly moulted 5th-stage larva showing nerve-supply.

L, thoracic muscle-fibre showing elongated mitochondria between the fibrils and double row of rounded mitochondria along each Z line (compare fig. 6, C).

glesworth, 1956) it was shown that the sheaths around the muscles in *Rhodnius* appear to be of the same nature as the basement membranes, the sheath enclosing the fat-body cells, and the perilemma around the ganglia and nerves. These membranes are largely, often wholly, the product of the amoebocytes. These cells contain PAS-positive inclusions which they discharge to form or add to the connective tissue membranes, composed apparently of some neutral mucopolysaccharide.

When the muscle-fibrils undergo involution the sheath becomes too large for the muscle and is therefore thrown into longitudinal folds. This is shown in transverse section in fig. 4, A and L. These connective tissue sheaths contain submicroscopic fibres (Baccetti, 1955) and therefore when seen in profile they appear birefringent. It is not surprising, therefore, that although the involuted muscles show no conspicuous birefringence, if they are separated from the cuticle they do show a weak birefringence. This is readily demonstrated by the use of a compensator. The muscle-sheaths then appear just perceptibly bright in the addition position; they show up clearly as dark bands on a grey background in the subtraction position.

This weak birefringence might be due to a few surviving muscle-fibrils. But in sections stained with Masson's trichrome stain, in which the sheath stains strongly with the light green and the muscle-fibrils stain red with the ponceau, I have been unable to detect any muscle-fibrils at this stage. Whether the birefringence is due wholly to a fibrous membrane seen in profile, or whether (as seems probable) there is some longitudinal orientation of the fibrous component in the sheath, which might render the membrane birefringent even in surface view, has not been determined.

Inside this sheath the nuclei are shrunken and inactive, with relatively small nucleoli. They are dispersed more or less irregularly. There is very little cytoplasm; but this contains mitochondria, both globular and filamentous in form, which are most plentiful immediately around the nuclei (fig. 6, A, p. 472).

Formation of Muscle-fibrils

After feeding, when the muscle-sheaths are stretched, the nuclei tend to arrange themselves in longitudinal rows. In the 1st-stage larva there is only a single row of 9–12 nuclei in each muscle (fig. 3, A). In the 4th stage, each muscle usually has three or four rows of nuclei, but these do not all run the whole length of the muscle.

1st-stage larva. Fig. 3, D–L shows the development of fibrils in the 1st-stage larva. By 2 or 3 days after feeding, birefringent fibrils are beginning to appear on each side of the row of nuclei. By 4 days the fibrils are increasing in number and in polarized light each muscle now appears in the form of two birefringent threads, one on each side of the isotropic core occupied by the nuclei (fig. 1, D); there is no striation at this stage. By 5 days (fig. 1, E) the muscles are thickening and traces of dark banding are beginning to appear, particularly at the periphery of the fibres. During the next few days the muscles increase rapidly in thickness as more fibrils are laid down, and striation becomes con-

spicuous and regular (fig. 3, H–K). By 10 days the muscles are fully formed (fig. 1, F); the isotropic core is still detectable but is less obvious. Moulting occurs during the night between the 10th and 11th days.

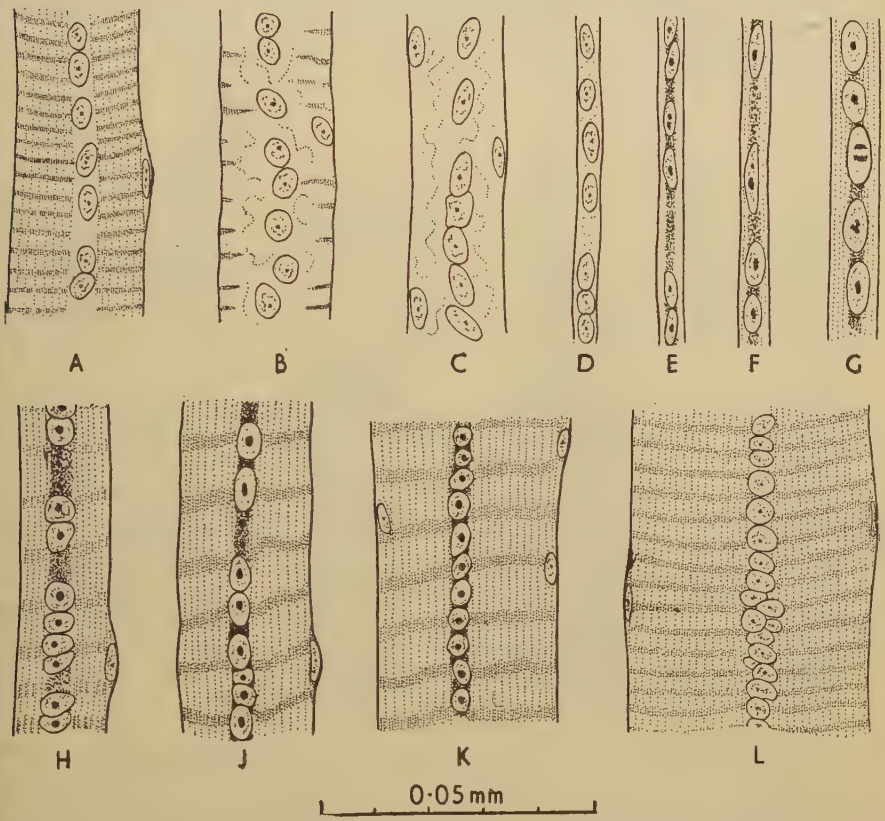

FIG. 3. Changes in the abdominal muscles of the 1st-stage larva after hatching and after feeding. A, immediately after hatching (compare fig. 1, B). B, 2 days after hatching. C, 4 days after hatching (compare fig. 1, C). D, 14 days after hatching immediately after feeding. E, 2 days after feeding. F, 4 days after feeding (compare fig. 1, D). G, 5 days after feeding (compare fig. 1, E). H, 6 days after feeding. J, 7 days after feeding. K, 9 days after feeding (compare fig. 1, F). L, 11 days after feeding (newly moulted 2nd-stage larva).

4th-stage larva. The formation of muscle-fibrils has been followed in the 4th-stage larva in whole mounts, and in transverse and tangential sections of the abdomen. At 2 days after feeding (fig. 4, B) the nuclei have enlarged and the cytoplasm within the sheath has increased, but as a rule no fibrils can be detected. By 3 days a large number of fine fibrils (estimated to be between 0·1 and 0·2 μ in thickness) have separated out in the cytoplasm (fig. 4, C). These fibrils often form a single layer disposed as a sheath around the core of nuclei, each fibril being separated from its neighbours by a space about equal to its own diameter (fig. 2, D).

Thereafter the muscles increase progressively in thickness (fig. 4, D–G). The individual fibrils appear to grow by intussusception, so that by 6–8 days they are perhaps 0·3–0·4 μ wide (fig. 2, E). These enlarged fibrils are quite widely spaced in the cytoplasm, they often appear flattened, and in the sections they may bend over at their cut ends (fig. 5, A). These cut ends may spread out slightly in a fan-like manner, and can then be seen to split into finer fibrils.

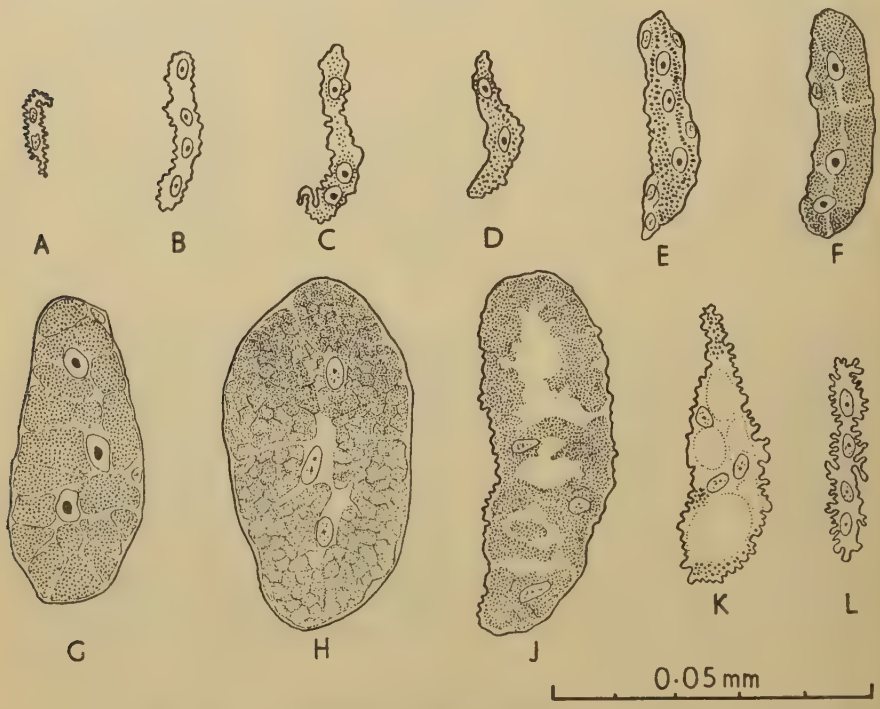

FIG. 4. Transverse sections of abdominal muscles of 4th-stage larva moulting to the 5th-stage. A, 4th-stage larva, 1 day after feeding. B, 2 days after feeding. C, 3 days after feeding, fibrils appearing. D, 4 days (compare fig. 1, G and H). E, 5 days, fibrils forming thicker bundles. F, 7 days (compare fig. 1, J). G, 10 days, showing greatly enlarged nucleoli (compare fig. 1, K). H, 14 days (newly moulted 5th-stage larva), nucleoli reduced (compare fig. 1, L). J, 2 days after moulting, vacuoles in the centre of the muscle. K, 4 days after moulting, fibrils persist only at the margins. L, 10 days after moulting, no fibrils remain; sheath highly folded. The peripheral nuclei in E and F are the nuclei of the nerve-sheath.

As this process continues the fibrils come to form the bulk of the muscle, and it becomes increasingly difficult to distinguish the individual fibrils in sections. But they are still more or less ribbon-like in general form, and although the fibril bundles are arranged in groups (Cohnheim's fields), if they are observed in cross-sections and the microscope is focused up and down, it is clear that the large fibrils are still splitting and anastomosing, often with fibrils in adjacent bundles, so that a given Cohnheim field changes its outline with the focus (see Tiegs, 1955).

When the fibrils are first formed, at 3–4 days after feeding, it is impossible to detect any striation in polarized light (fig. 1, G and H). A somewhat irregular striation begins to appear at 5 days, and thereafter becomes increasingly conspicuous as the muscle grows in thickness. At the same time the striation in adjacent fibrils becomes progressively better aligned; and in the fully formed muscle at 12–13 days after feeding the striations are usually continuous right across the muscle (fig. 1, L).

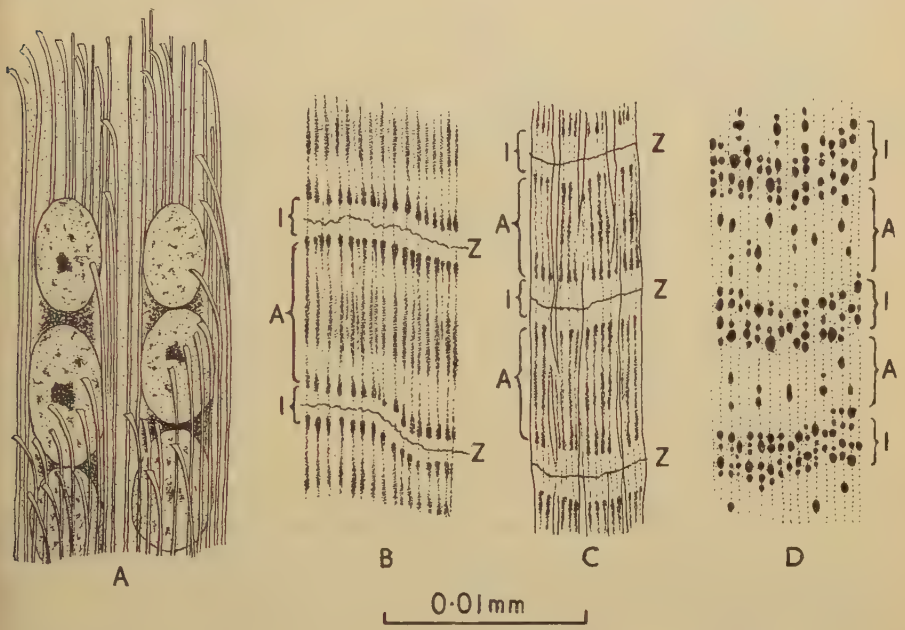

FIG. 5. A, longitudinal section of abdominal muscle of 4th-stage larva 6 days after feeding, showing fibrils thickening but still well separated. B, longitudinal section of muscle at 13 days after feeding, showing fully developed striation. Masson's trichrome stain. C, the same in newly moulted 5th-stage larva. Bromothymol blue to show protein density. D, the same; neotetrazolium method for succinoxidase.

In whole muscles fixed in Carnoy's or Bouin's fixative and stained with Hansen's trioxyhaematein, striation becomes visible at just the same time as in polarized light. It takes the form of a rather indefinite dark band in the isotropic region. The telophragma (Z line) does not stain. The development of the telophragma is most readily followed in preparations fixed in osmium tetroxide and stained with Hansen's trioxyhaematein. It first appears, at 5–6 days after feeding, in the form of minute black points irregularly dispersed throughout the muscle. At 7 days these points are becoming aligned across the fibre and can be recognized as the telophragma. By 9 days the structure is fully formed and appears as a sharp black line extending across adjacent fibrils, with a very narrow zone of diffuse black staining on each side of it. The distance between successive Z lines before moulting occurs is about 12 to 13 μ. After moulting, when the muscles contract somewhat, it is about 9 to 10 μ.

Immediately after feeding, as we have seen (fig. 6, A), the mitochondria are irregularly distributed, but most plentiful around the nuclei. By 2 days after feeding they are more numerous, there are many elongated forms and many of these are becoming orientated in the long axis of the muscle. At 3 days this change is more evident: the plentiful mitochondria in the axial core of cytoplasm between the nuclei are often rounded and quite irregularly arranged.

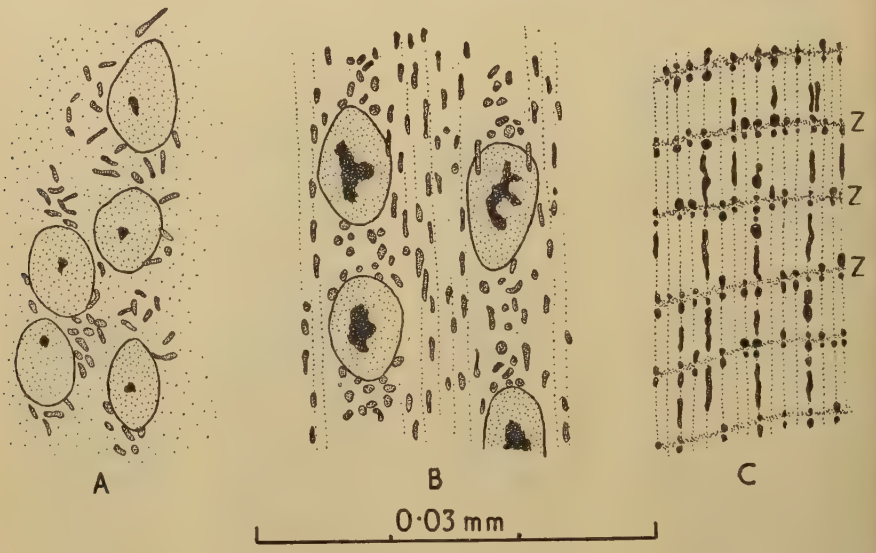

FIG. 6. A, part of muscle in 4th-stage larva immediately after feeding, showing inactive nuclei with irregularly arranged mitochondria. B, the same 3 days after feeding, showing active nuclei with enlarged nucleoli; abundant mitochondria, many of them lying longitudinally between the newly forming fibrils. C, fully formed muscle from the thorax showing elongated mitochondria between the fibrils and rounded forms on each side of the Z line (compare fig. 2, L).

Elsewhere the mitochondria lie between the newly forming fibrils and many of them are compressed into an elongated form (fig. 6, B). This state of affairs continues until 6 days after feeding; but thereafter the fibrils are becoming so numerous and densely packed that it has not been possible to observe the mitochondria. Fig. 6, C shows the mitochondria in a fully formed muscle from the base of the leg. They lie irregularly distributed between the fibrils, very variable in form and size, but many of them greatly elongated. In addition, there is a concentration of rather small mitochondria ('J granules') on each side of the Z line (compare fig. 2, L).

In sections of the fully developed muscles stained with Masson's trichrome stain, the continuous fibrils staining with the ponceau are crossed by a deep-purple-staining telophragma which, as usual, runs across the whole muscle and is attached to the muscle-sheath. On each side of this there is a relatively pale region, and then a long zone (the A disk) in which a blue-grey coloration may obscure the red staining of the fibril (fig. 5, B). A very similar arrangement

is seen in fibrils stained with bromothymol blue to show protein density (Mazia, Brewer, and Alfert, 1953) (fig. 5, C); in this figure the anastomosis of adjacent fibrils can be seen (compare Edwards and Ruska, 1955); they are now about 0·8 μ across. Thus in their fully formed state these muscles show a fairly elaborate degree of striation of standard type.

Changes in the Muscle-Nuclei and Distribution of Ribonucleic Acid (RNA)

The changes in the muscle-nuclei under the influence of the moulting hormone will be described elsewhere. It may be said here that in the resting insect these nuclei are small, with a relatively inconspicuous nucleolus and a nuclear membrane which is pale staining and (in fixed preparations) often crumpled. The cytoplasm around is almost non-existent. Within 6 hours after feeding the nuclei are becoming active and the nucleoli enlarging. By 24 hours after feeding these changes are conspicuous: the nucleus is now tense and vesicular, the nucleolus is greatly enlarged and rich in RNA, and so is the bounding membrane of the nucleus. The cytoplasm is beginning to increase in amount and, particularly in the neighbourhood of the nuclei, it contains much RNA. These changes can be seen in the muscles of the 1st-stage larva in fig. 3, E. Mitosis in the muscle-nuclei begins 2 or 3 days after feeding; it is coming to an end (in the 4th-stage larva) by 7 days.

At the 3rd or 4th day after feeding, when the fibrils are beginning to separate out, the nuclei are very large and the nucleoli and the cytoplasm of the nuclear core contain abundant RNA. In addition, there are elongated deposits and granules of RNA between the newly formed fibrils. The fibrils themselves show no nucleic acid staining—they appear as glassy rods between the RNA deposits (fig. 7, A and B).

During the 5th and 6th days, when the striation is beginning to appear, these interfibrillar deposits of nucleic acid are becoming aggregated at the level of the I bands. Fig. 7, C shows a section of a muscle at 8 days, stained with pyronin and methyl green. The dark staining in this figure is due wholly to RNA; it takes the form of granules between the fibrils; some of these granules are readily seen with the light microscope, but there are others so fine as to merge gradually into an apparently diffuse staining. There are still massive deposits of nucleic acid in the cytoplasmic core between the nuclei and there are occasional granules between the fibrils outside the I bands.

After treatment with ribonuclease these bands and the other deposits staining with pyronin and gallocyanin are completely removed and the nucleoli no longer stain with these dyes; but that makes no detectable difference to the isotropy of the I bands and these will still stain (though not so strongly as before) with Hansen's trioxyhaematein. This staining with haematoxylin reveals no distinct structural difference in the I band, but it sometimes gives the impression that there is a fusiform thickening of the fibril at this level with material staining with haematoxylin.

A comparison of the preparations stained for ribonucleic acid (fig. 7) with those showing the mitochondria (fig. 6) suggests that the mitochondria are probably the source of many of the granular deposits of RNA, but that in addition, there is a large amount of RNA in the nuclear core of the muscle and in the I bands which appears diffuse in the light microscope and is probably associated with cytoplasmic reticulum.

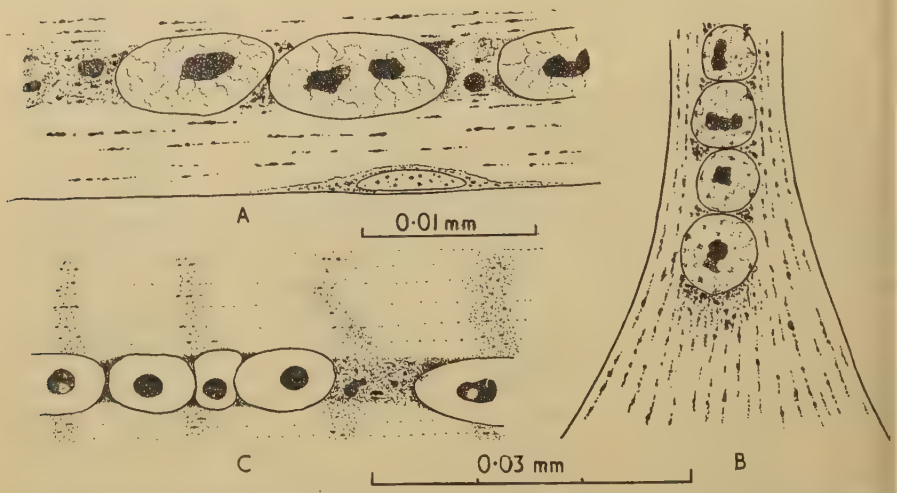

FIG. 7. A, part of muscle from 4th-stage larva 4 days after feeding, stained with gallocyanin at pH 1·6 to show nucleic acid. B, end of muscle of 1st-stage larva 4 days after feeding, similarly stained. Nucleic acid between the nuclei and between the fibrils. C, part of muscle from 4th-stage larva 8 days after feeding, stained with pyronin and methyl green. The darkened areas indicate the distribution of ribonucleic acid (pyronin staining).

The enormous enlargement of the nucleoli, and the deposits of RNA between the nuclei, persist until the day before moulting (figs. 3, K; 4, G). All this time the fibrils appear to be increasing in number. But on the day of moulting the nucleoli are markedly reduced (figs. 3, L; 4, H), the nuclei are smaller, and the conspicuous deposits of RNA have disappeared from the cytoplasm between them.

Involution of the Muscles

1st-stage larva. Fig. 3, A shows the appearance of a sternal muscle of a 1st-stage larva on the day of hatching from the egg. By 2 days after hatching (fig. 3, B) striated fibrils persist only at the periphery of the muscle. By 4 days (fig. 3, C) all trace of fibrils has disappeared.

5th-stage larva. Fig. 8, A shows a portion of a sternal muscle in the 5th-stage larva immediately after moulting, with the striations aligned right across the muscle. At 1 day after moulting there is almost no change, though perhaps the striations do not stain quite so strongly.

At 2 days after moulting, striation is still evident in stained preparations and

in polarized light. The muscles are becoming reduced in thickness and the birefringence is weaker. Vacuoles of varying size are appearing in the muscle core between the nuclei (figs. 4, J; 8, B). Droplets of fat are present in the muscle at this stage.

At 3 days, fibrils and striations are still distinct in polarized light, but in stained preparations the banding is becoming faint. The fibres are now

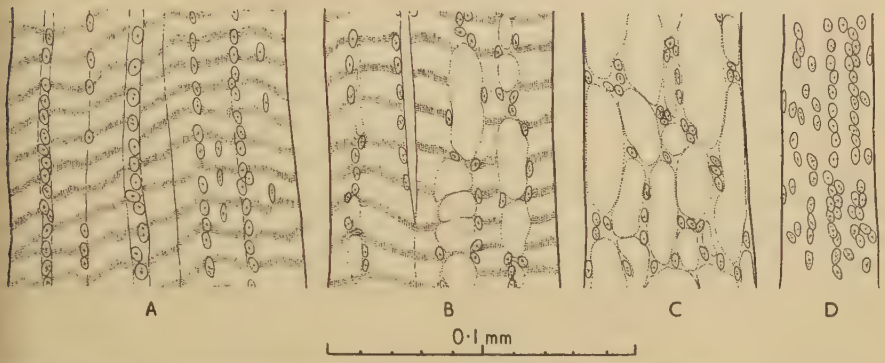

FIG. 8. A, part of abdominal muscle in newly moulted 5th-stage larva, stained with Hansen's trioxyhaematein (compare fig. 4, H). B, the same, 2 days after moulting (compare fig. 4, J). C, the same, 4 days after moulting (compare fig. 4, K). D, the same, 10 days after moulting (compare fig. 4, L).

highly vacuolated, most of the muscles being reduced to vacuolated sacs with irregularly dispersed nuclei connected by cytoplasmic strands enclosing the vacuoles.

At 4 days, the weak birefringence that persists throughout most of the muscle is probably due solely to the folds that are now developing in the muscle-sheath (p. 468). The greater part of the muscle contains nothing but vacuoles (fig. 8, C). A few striated fibrils still remain along the margins of the muscles near their insertions (fig. 4, K).

At 10 days after moulting the muscles are reduced once more to highly folded sheaths containing irregular strings of nuclei and very little cytoplasm. No muscle-fibrils remain (figs. 4, L; 8, D).

In some insects the phagocytic amoebocytes in the blood are said to play an active part in removing the tissue debris that results from the autolysis of muscles during metamorphosis. They are not concerned in the process of involution of the abdominal muscles in *Rhodnius*. They are excluded from the muscle by the fibrous sheath, and there is no accumulation of these cells in the neighbourhood of the autolysing muscles.

Development of Succinoxidase

The neotetrazolium method of Shelton and Schneider (1952) was applied to a series of 4th-stage larvae at intervals after feeding. The freshly dissected sternites were incubated in the neotetrazolium mixture at 37° C for 45 minutes, rinsed in Ringer's solution, mounted in glycerine jelly, and

examined at once. Controls carried out on the same material in the absenc of succinate were always negative.

There is no reaction when the intact muscles in the fully developed state ar incubated in this mixture: it is necessary to cut through the muscle-sheath Fig. 2, F shows the absence of any reaction in the sternal muscles on the lef side, where the muscles are intact, but an intense positive reaction in th contracted stumps of the cut muscles on the right side.

The thoracic muscles and the dorso-ventral muscles of the abdomen alway show a strong positive reaction. But the reaction is practically negative in th cut muscles of the abdominal sternites during the first few days after feeding On the 5th day a few scattered purple granules may appear in some of th muscles. On the 6th day the reaction is quite definite. From then onwards i increases in intensity up to the day of moulting.

As the reaction develops the purple granules of the formazan show a charac teristic distribution. They occur in rows between the fibrils, just like th mitochondria; and they are much more abundant in the isotropic disk, so tha the striation of the muscle shows up conspicuously (fig. 2, G, H; compar fig. 5, D). There is also a faint pink staining of the substance of the isotropi disk—which suggests the presence of lipid material in this region. In fac the granules show just the same distribution as the mitochondria (figs. 2, L; 6, C Rutenberg, Wolman, and Seligman (1953) describe a similar distribution o granules between the fibrils in mammalian muscle, but the concentration i the anisotropic disks was not so evident as in *Rhodnius* muscle.

The Nadi reaction has exactly the same distribution as the reduction o neotetrazolium. It is much more evanescent, but when examined at the righ moment the muscles again show rows of interfibrillar blue granules concen trated in the I bands.

During the involution of the sternal muscles, the reaction remains intens in the cut muscles at 1 day after moulting. By 2 days after moulting th granules are still related with the striation, but the intensity of the reaction i reduced to about half. At 3 days after moulting, the reaction is almost com pletely negative, though a faint pink staining of the I bands remains.

Development of Contractility

When the fully developed sternal muscles are cut through, they rapidl contract down to stumps not more than 10–15% of their initial length. Th development of this property has been studied by removing the sternite cutting across the cuticle and muscles in the middle of each segment on on side of the mid-line, and examining at once in Ringer's solution with polarize light.

In the involuted state the muscles do not contract after cutting. The same i true during the first 3 days after feeding. On the 4th day, when the birefrin gent fibrils are becoming quite conspicuous, the ends of the cut muscles sti remain almost up to the margin of the cut cuticle. At the most they contrac down to 90–95% of their initial length. On the 5th day, when striations ar

beginning to appear, the amount of contraction is extremely variable: some of the muscle bands may contract to 40% of their length, others contract only to about 60%. Thereafter the contractility increases progressively: on the 7th day, most muscles contract to about 40% of the original length, a few contract down to 33%. During the 8th, 9th, and 10th days most muscles contract down to about 33%; at 12 days they contract at once to less than 25%; and in the fully developed muscle at 14 days they contract rapidly to 10–15% of the initial length.

Thus, spontaneous contraction of the cut muscles does not occur until the isotropic bands first appear in the fibrils at the 5th day after feeding. As the muscles thicken, contraction becomes more intense.

At the time of moulting, as we have seen, the muscles shorten considerably; but when cut they still contract down to about 30–40% of their initial length. One day after moulting they contract to about 50–60%. At 2 days, not quite so much— say 60–70%. At 3 days after moulting, when, as we have seen, the muscles are filled with vacuoles, though striation persists in the fibrils which remain, the cut muscles show slight irregular twitching but no shortening.

Innervation of Muscles

The abdominal ganglia in *Rhodnius* are fused with the ganglia in the thorax. They give off a fan-like array of nerves to the abdomen, and these in turn give rise to branches which run transversely across the middle of the sternites at right angles to the sternal muscles (fig. 2, K). In the neighbourhood of each muscle these nerves give off small branches which ramify all over the surface of the muscle and, as is usual in insects, they end in clusters of fine branches in every part of the muscle; there is no sign of an end plate (compare Meyer, 1955).

The transverse nerves to the muscles, and the branches which ramify on the muscle-sheath, are enclosed, as usual, in a perilemma with a cellular perineurium beneath. The perineurium is always rich in succinoxidase, so that in preparations in which the neotetrazolium method is applied to the intact muscles, the course of the nerve-branches is marked out by the purple granules which accumulate in the perineurium (fig. 2, J).

Fig. 9, A shows the middle portion of a sternal muscle in the fully developed state in a newly moulted 5th-stage larva, with the rich nerve-supply stained with methylene blue. Fig. 9, B shows a similar muscle 10 days after moulting, when the muscle-fibrils have completely degenerated. The muscle-sheath is collapsed and shrunken but the nerves persist apparently unchanged.

It was shown by Kopeč (1923) that the thoracic muscles in the adult *Lymantria* do not develop if they are deprived of their innervation by removal of the thoracic ganglia in the young pupa (compare Nüesch (1952) on *Platysamia*). It was therefore of interest to see whether the innervation of the sternal muscles in *Rhodnius* is necessary for the formation of fibrils. Fourth-stage larvae at 24 hours after feeding were injected with 1 μg of the moulting hormone 'ecdyson' of Butenandt and Karlson (1954) (for the gift of which I am

indebted to Dr. P. Karlson) and the abdomen was then ligatured and separated from the nerve-cell bodies in the thorax. Moulting is induced in about 2 weeks in the isolated abdomen (Wigglesworth, 1955*b*). The sternal muscles

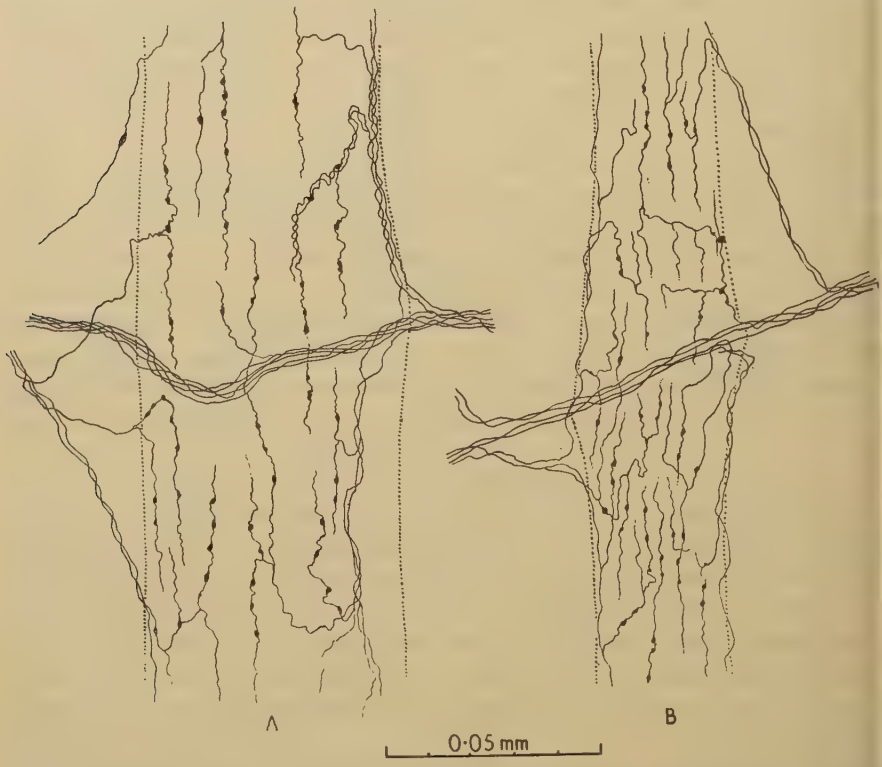

FIG. 9. A, middle portion of abdominal muscle of newly moulted 5th-stage larva, showin nerve-supply stained with methylene blue. B, the same, 10 days after moulting.

show some distortion but otherwise they are well developed and show th normal striation. Clearly these muscles are not dependent on their nerve supply for the deposition of fibrils.

DISCUSSION

In many insects there are striking changes in the muscular system, with th dissolution of existing muscles and the formation of new ones, during de velopment of the pupa or the adult. But repeated cycles of development an involution in the same muscles do not seem to have been described before. may be that this phenomenon is widespread in insects. Sternal muscles ar well developed in all segments of the abdomen in the newly hatched bed-bu *Cimex*; those on the anterior segments show no obvious change, but th muscles on segments 6, 7, and 8 disappear within 3 or 4 days. In *Dysderc* (Pyrrhocoridae) sternal muscles are vestigial in the abdomen except in seg ments 5, 6, and 7, where they are well developed in the newly moulted 5th

stage larva. One day after moulting they are becoming much less birefringent and have usually disappeared by 3 or 4 days after moulting.

The formation of the muscles in the embryo of *Rhodnius* has not been studied; but throughout subsequent growth the nuclei of the intersegmental muscles are cut off from the cells of the body-cavity by the muscle-sheath. They increase in number by mitosis during each moulting cycle; they are not replenished by 'mesenchyme cells' entering from outside, as described by Debaisieux (1954) in Crustacea.

The fibrils appear to 'crystallize out' as continuous threads between the longitudinally disposed mitochondria. In the earliest stage at which clearly stained fibrils have been seen (at 3 days after feeding), they measure only 0·1–0·2 μ in diameter. Thereafter they grow, as has been shown often enough in vertebrates (see Schmidt, 1937), by intussusception and longitudinal splitting. The fully formed fibril has a diameter of about 0·8 μ. As in embryonic muscle-cells (Vincent, 1955) the nuclei have greatly hypertrophied nucleoli throughout the period when new fibrils are being formed.

When they first appear the fibrils are uniformly birefringent; the periodic isotropic regions are added later. That has been described in vertebrates by Schmidt (1937), Le Gros Clark (1946), and others. The Z line seems to appear after the isotropic banding has formed—but that point has not been sufficiently studied.

There is no information about the factors which initiate the break-down of the muscle-fibrils after moulting. It is independent of the nerve-supply and is presumably controlled by humoral means—as is the break-down of certain organs at the conclusion of metamorphosis (Wigglesworth, 1955*a*). The process of break-down is a pure autolysis which starts around the nuclei in the core of the fibres and spreads outwards. That is in agreement with the observations of E. Schmidt (1919) on *Psychoda*. In the break-down of many insect muscles at metamorphosis the phagocytic blood-cells play a more or less important part (Lange, 1932; Blaustein, 1935). In *Rhodnius* they seem to play no part at all and do not even collect around the outside of the autolysing muscles.

The development of contractility has been studied only by the crude method of noting the spontaneous shortening after section. It coincides with the appearance of striation. The distribution of succinoxidase, which is concentrated in the mitochondria (sarcosomes), agrees with the biochemical findings of Watanabe and Williams (1951) and the histochemical observations of Wachstein and Meisel (1955). It is characteristic of insect muscles that besides the scattered interfibrillar mitochondria there is a concentration of small globular mitochondria (the 'J granules') on each side of the Z line (Jordan, 1933). As a result, the *Rhodnius* muscles treated by the neotetrazolium method show well-marked banding in the I disks. It is perhaps worth noting that even in the resting or involuted state the muscles contain plenty of mitochondria; but the neotetrazolium test does not become positive until some 5 days after feeding, when contractility is beginning to develop.

Gerendas and Matoltsy (1948) (quoted by Perry, 1955) have ascribed the

relative isotropy of the I band to the negative birefringence of a nucleoprotein contained in this region. The sternal muscles in *Rhodnius* certainly show well-marked bands of ribonucleic acid in the I disks; but the removal of this with ribonuclease makes no difference to the appearance of the fibres in polarized light. Much of the ribonucleic acid is concentrated in the mitochondria, which have the same distribution. It is impossible to say at the moment how much of the nucleic acid in the bands is in the mitochondria and how much is in the cytoplasmic reticulum.

During the cycles of muscle-growth and involution the nerve-supply appears to remain unchanged, apart from an equivalent amount of growth. It would be interesting to know just how the nerve-endings are connected to the contractile fibres. No end-plates have been detected in insect muscles.

It is hoped to continue the study of these muscles with the electron microscope, for this material should provide a favourable opportunity to observe the first appearance of muscle-fibrils and their subsequent differentiation; and the great length of the sarcomeres (10–12 μ) may provide opportunities for obtaining further information on their fine structure.

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Some Observations upon the Golgi Elements of the Cells of the Surgically Removed Human Anterior Pituitary

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With one plate (fig. 1)

Summary

1. The lipid inclusions of the cells of the pars distalis of the human pituitary are not restricted to the zone occupied by the Golgi element.

2. No evidence was obtained to suggest that a canalicular system is invariably associated with the Golgi zone. The frequency with which such systems were observed appeared to depend on the mode of fixation and other factors.

Introduction

DURING the course of an investigation into certain aspects of the general cytology of the pars distalis of human pituitaries removed and fixed in the operating theatre, the opportunity was taken to examine the Golgi elements of the glandular cells. Certain observations, principally of a topographical kind, have already been briefly reported upon elsewhere (Foster, 1956). In the following short report, attention has been focused upon the relationship of the argentophil structures seen after the application of the Aoyama technique to (1) the intracellular sudanophil lipid inclusions, and (2) the so-called 'negative images' of the Golgi elements.

Materials and Methods

Ten human pituitaries were available for this study. They were obtained from women with mammary carcinoma and were generally removed as fragments of varying size, which were immediately fixed. In most instances, the fixation was good and, as has been reported elsewhere (Foster, 1956), apart from localized carcinomatous infiltration in two instances, the bulk of the cells observed could be regarded as normal.

In addition, one pituitary obtained $2\frac{1}{2}$ hours after death was examined for purposes of comparison.

The material was treated as follows:

Fragments were:

(1) Fixed in Baker's formaldehyde-calcium and embedded in gelatine. Three-micron frozen sections were mounted in Farrants's medium and examined unstained by phase-contrast microscopy. Others were either coloured with Sudan black, mounted in Farrants's medium, and examined by ordinary and phase-contrast microscopy, or stained by the PAS / metachrome yellow method (Foster, 1956).

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(2) Fixed in 10% neutral formalin; paraffin sections were stained by th Azan method.

(3) Fixed in Helly's fluid and treated as above. Some unstained sectior mounted in Farrants's medium were examined by phase-contrast microscopy

(4) Fixed in Regaud's fluid overnight, embedded in gelatine; 3-μ sectior were examined unstained by phase-contrast microscopy.

(5) Fixed as above, but postchromed for 5 days and embedded in paraffir Five-micron sections were stained by Altmann's method for mitochondria.

(6) Fixed in Champy's fluid; paraffin sections were examined unstained b phase-contrast microscopy.

(7) Fixed in Aoyama's fluid and impregnated with silver. Three-micro frozen gelatine-embedded sections were toned with gold chloride, mounte in Farrants's medium, and then treated as follows:

(*a*) Photographs were taken of a number of suitable fields showing goo impregnations of the Golgi element.

(*b*) The metal was then removed by treatment with Lugol's iodine followe by immersion in sodium thiosulphate (Lacy, 1954*b*). It was found tha in gelatine-embedded sections the complete removal of the gold wa difficult to achieve. There were always, however, substantial areas of section in which removal was complete. After washing in water an mounting in Farrants's medium, the same areas were examined b phase-contrast microscopy and rephotographed.

(*c*) The sections were coloured with Sudan black to reveal the lipid inclu sions and mounted in Farrants's medium; the areas under study wer again examined and photographed.

(8) The material was treated as in (7) except that it was embedded i paraffin and was mounted in Canada balsam.

Results

A careful study by phase-contrast microscopy of thin frozen sections surgically removed anterior pituitary tissue which had been fixed in Baker formaldehyde-calcium, embedded in gelatine, and mounted in Farrants medium readily showed (*a*) the intracellular secretion granules of the chr mophil cells as small, dark, separate bodies, and (*b*) the vacuolated intracellul lipid inclusions as light circular areas with a complete or incomplete da rim of variable thickness. The smaller solid lipid inclusions were not di tinguishable from the secretion granules. The study failed, however, to reve any structures comparable in form, position, or extent with the black ne works so commonly seen after similar material had been prepared by Aoyama silver impregnation method. Occasionally, an irregular and delicate canalicul system of indefinite extent could be seen, but there was no doubt that th appearance resulted from a purely fortuitous arrangement of intergranul channels. Similar material coloured with Sudan black revealed abunda sudanophil intracellular lipid bodies, but neither direct nor phase-contra microscopy demonstrated any structure comparable in form or position wi

the argentophil networks obtained by the Aoyama method. However, in similar sections prepared by the PAS / metachrome yellow method to show the chromophil cell-types and mounted in Farrants's medium, a mesh of what appeared to be clear canals similar in position and extent to the argentophil nets was observed in a very small proportion of the cells. In the post-mortem specimen used for comparison, only on one occasion was a comparable apparent canalicular system seen in a section from tissue fixed in Aoyama's fluid embedded in gelatine and examined by phase-contrast microscopy (without silvering).

It is well known that in stained paraffin sections of animal and human post-mortem pituitary tissue, pale intracellular canalicular systems appearing to correspond with the argentophil or osmiophil networks of the classical Golgi element are sometimes seen. Some of the surgical material was examined from this point of view. In particular, thin paraffin sections of the following material were studied: (*a*) fixed in neutral formaldehyde, stained by the Azan method; (*b*) fixed in Helly's fluid, stained by Azan or PAS / orange G; (*c*) fixed in Regaud's fluid, postchromed, stained by Altmann's acid fuchsin. Pale canalicular systems commonly referred to as 'negative images' of the Golgi elements were seen in all three instances, but, even in the Regaud-fixed material where they were most frequent, they were present in only a small proportion of the cells (fig. 1, B). In this material also, it was noted that when focusing up and down upon the nuclei, an impression of an intranuclear canalicular arrangement was often obtained, and this was presumably an optical artifact. In material fixed in Regaud's fluid, embedded in gelatine, but not subjected to post-chroming, the study by phase-contrast microscopy of unstained sections or ones coloured with Sudan black failed to demonstrate any system of canals unequivocally comparable with the argentophil nets.

Thin paraffin sections of tissue which had been fixed in Champy's fluid were studied with the phase-contrast microscope, but 'negative images' were not observed. The lipid inclusions were strongly blackened.

An examination of 3-μ frozen sections of material prepared by the Aoyama method and embedded in gelatine showed, in two of the fragments from five pituitaries treated in this way, good impregnations of the Golgi elements. That is to say they conformed to the convention that, in most vertebrate somatic cells, the criterion of a satisfactory metallic impregnation is the demonstration of a black network in the cytoplasm. Study of the nets after toning the sections with gold chloride did not reveal any evidence for the presence of non-argentophil channels or cavities within the constituent argentophil strands, but, of course, the ability or otherwise to see such structures might well have depended upon the degree of impregnation. The relationship between the configuration of the Golgi elements and the cell types has been discussed elsewhere (Foster, 1956) and is not a matter immediately relevant to the present investigation.

When the metallic impregnation was removed by the technique previously described and chosen groups of cells were re-examined by phase-contrast

microscopy, diligent search failed to show any canalicular systems in the places formerly occupied by the argentophil material (fig. 1, C–H). A study of the remaining cells of the sections outside the selected groups did, occasionally, show a system of apparent canals resembling the 'negative images' which might well have corresponded with the argentophil networks. One such example is illustrated in fig. 1, A.

Finally, the same areas were examined after the sections had been coloured with Sudan black, and here the numerous sudanophil lipid inclusions were readily seen (see also Foster, 1956). It was observed that the distribution of these bodies was in no way related to that of the argentophil material, since it appeared to be quite random (fig. 1, C, D, E). When the sudanophil bodies were found in the zone of the Golgi element, as they sometimes were, they were not restricted to it. It was also noticed that in some cells there were areas containing sudanophil material in the region formerly occupied by the Golgi element (fig. 1, G). This sudanophilia, which was associated with small granules and sometimes threads, appeared to be non-specific and to be associated with incomplete removal of the gold by the bleaching treatment, since, in sections deliberately only partially bleached, similar sudanophilia was much more common. Again, 'negative images' were infrequently seen either by direct or phase-contrast microscopy.

Thin paraffin sections of a surgically removed pituitary and of the ones obtained after death, prepared by the Aoyama technique and subjected to the procedures mentioned above, again gave similar results. In these, however, the sudanophilia of the lipid inclusions had virtually disappeared as had that of the Golgi zones. When these sections were examined unstained by phase-contrast microscopy, three mounting media of differing refractive indices were successively used on the same section (Farrants's medium, liquid paraffin, and neutral Canada balsam). It was observed that the few 'negative images' seen were most readily determined when Canada balsam was used as a

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FIG. 1 (plate). A, surgically removed human anterior pituitary cells, prepared by Aoyama technique and mounted in Farrants's medium after removal of the metallic impregnation. 3 μ. Phase contrast. To the left of the nucleus in the cell (*g*) is what is probably the 'negative image' of the Golgi element—one of the few examples seen in preparations of this kind.

B, surgically removed human anterior pituitary cells. Prepared by the Regaud method, embedded in paraffin wax, and stained by Altmann's acid fuchsin. 5 μ. A definite 'negative image' is seen in the cell marked *g*.

C, surgically removed human anterior pituitary cells (the two large cells are α-cells). Prepared by the Aoyama method to show Golgi elements. Embedded in gelatine. 3 μ.

D, the same field as C after colouring with Sudan black. The lipid bodies are not specifically related to the Golgi areas.

E, the same field as C after removal of the metallic impregnation. Phase contrast. No canalicular systems are evident in the Golgi regions.

F, surgically removed human anterior pituitary cells. Prepared by the Aoyama method to show Golgi elements. Embedded in gelatine. 3 μ.

G, the same field as F after colouring with Sudan black. There is a sudanophilia in some of the Golgi zones, but the lipid inclusions proper are not restricted to these zones.

H, the same field as F, after a somewhat incomplete removal of the metallic impregnation. Phase contrast. No canals are seen in the Golgi regions. The small intracellular spaces represent portions of the lipid inclusions.

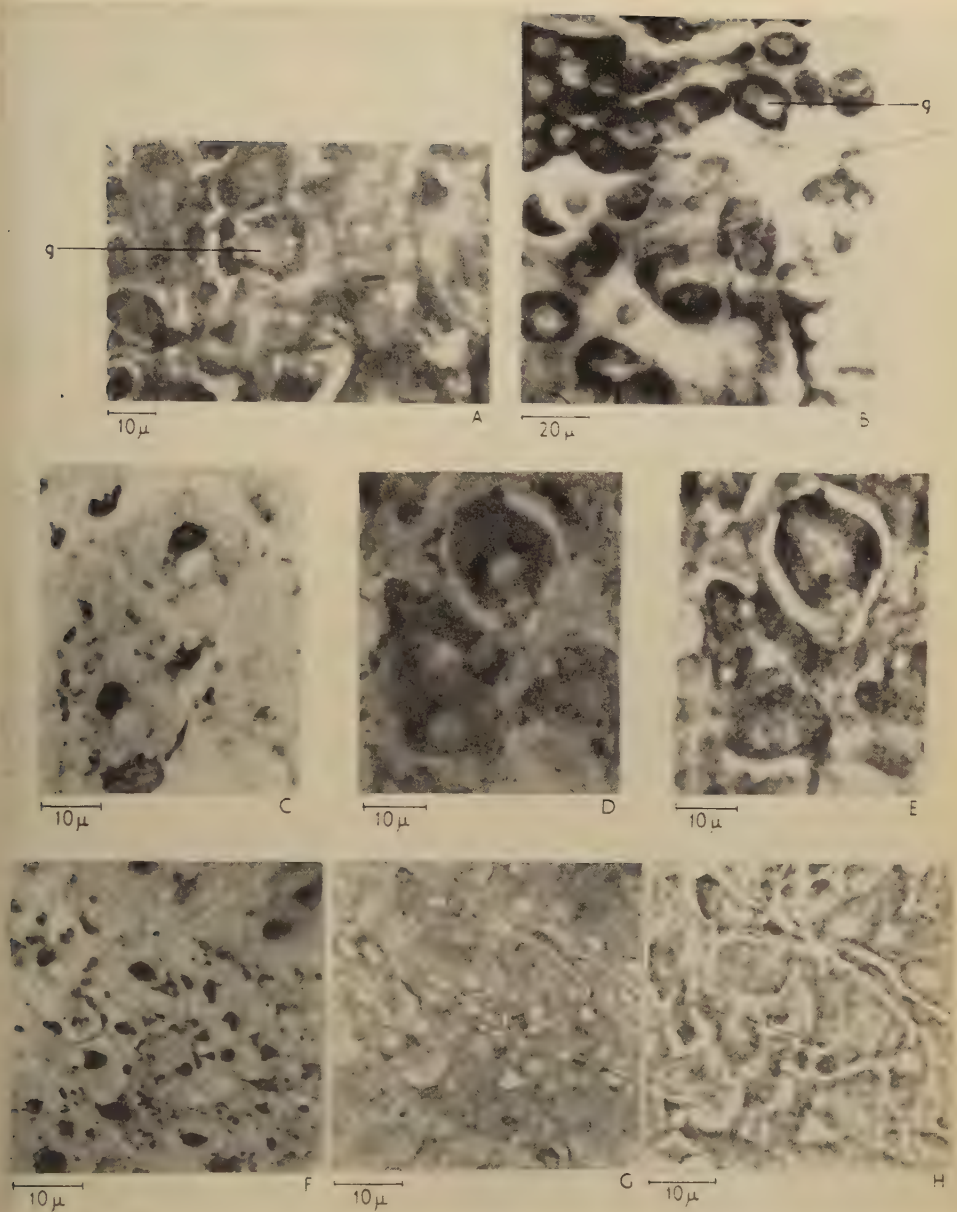

FIG. 1

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mountant. It may not be out of place to mention here, that in connexion with quite a different problem, paraffin sections of human anterior pituitary tissue fixed in Helly's fluid were examined by phase-contrast microscopy when mounted in a series of media with refractive indices ranging from 1·481 to 1·656. (These were prepared by mixing liquid paraffin and monobromonaphthalene in different proportions.) In no instance were 'negative images' seen any more frequently than has already been reported above.

Discussion

The study of the Golgi area in the *fixed* somatic cells of vertebrates by direct and phase-contrast microscopy has resulted in the recognition of three principal sorts of inclusion:

(1) a canalicular system or so-called 'negative image' of the Golgi element, which has commonly been equated with canalicular systems reported in living cells;
(2) lipid bodies;
(3) argentophil or osmiophil elements, often in the form of nets.

Of these, only the last can be regarded as being in any degree constant.

Inclusions (1) and (2) have sometimes been described as occurring together (Lacy, 1954*a*, *b*, and *c*). Furthermore, certain authors, among them Baker (1949) and Thomas (1947, 1948), have identified at least some of the vacuolated lipid bodies with the neutral red droplets of the living cell.

In the present investigation negative images were certainly seen in a small proportion of cells after various methods of fixation, and these occurred in those regions of the cytoplasm where an argentophil network would probably have been seen after the Aoyama technique. It seems worthy of note, however, that canalicular systems were only noticed in any significant numbers after fixation in Helly or Regaud's fluids followed by embedding in paraffin. By contrast, a technique such as fixation in formaldehyde-calcium followed by embedding in gelatine, which could presumably be regarded as less drastic, produced virtually no evidence for such systems. The only exception to this was the appearance of canals in a few cells in sections prepared as above, but subsequently treated by the PAS procedure to demonstrate mucoprotein. Allowing for the fact that not every cell will be cut through its Golgi region, there would certainly have been more Golgi zones theoretically visible than the number of 'negative images' actually seen.

When in thin sections of material prepared by the Aoyama method and embedded in gelatine, the metallic deposits representing the Golgi element were removed by iodine treatment and examined either unstained or coloured with Sudan black, canals were only occasionally seen. Had the silver been deposited in close relationship to a pre-existing arrangement of canals, many more 'negative images' should have been evident in the sections.

Lacy (1954*a*, *b*, and *c*), working on the exocrine and endocrine cells of the pancreas of mouse and man, has described canals in the Golgi zones of both

kinds of cell after fixation by Baker's method and the examination by phase-contrast microscopy of sections embedded in gelatine. Only in the human islet cells does he indicate that the appearance of such structures was infrequent. By contrast, Casselman and Baker (1955), investigating the sympathetic neurones of immature rabbits with the same techniques as were used by Lacy, state: 'A careful study of these preparations has not revealed any canalicular object resembling what Lacy reports in the pancreatic cell.'

The only conclusion to be drawn from the writer's observations, which are clearly at variance with those of Lacy on the pancreas, is that under certain conditions—primarily of fixation, perhaps—canalicular objects are evident in a very small proportion of the cells. This could be explained by assuming (*a*) that where they occur the canals do in fact correspond with the argentophil nets, but that the remaining, and by far the larger proportion, of the nets correspond with some other entity, reticular in form yet non-canalicular in nature, or (*b*) that there is an entity always present within the Golgi area of the fixed cell which, although it generally shows an affinity for silver or osmium, manifests itself as a canalicular system only under certain conditions. Of the two hypotheses, the latter seems the more satisfactory, and could, moreover, be in accord with the results of recent studies of the Golgi zone by electron microscopy (Dalton and Felix, 1955). In addition to small granules, these have shown, in a fairly wide range of cells, the presence of vacuoles associated with a system of double membranes. Of the latter elements, the membrane systems would seem to be, *a priori*, the components least likely to have been induced secondarily by fixation or other unphysiological procedures and might well be the structures upon which the silver or the osmium of the classical impregnation techniques is primarily deposited.

I would like to thank Mr. H. Long for his technical assistance.

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The Structure of the Germinal Epithelium of the Fowl Testis with Special Reference to the Presence of Multinuclear Cells

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With one plate (fig. 1)

Summary

The effects of histological fluids on the cells of the germinal epithelium of the fowl testis have been studied with a view to confirming the fact, evident from studies of living cells, that the formation of multinuclear cells in the process of spermatogenesis is a normal occurrence. It is considered that in the most rapidly dividing germ-cells the cytoplasm is not in a very concentrated state and the usual methods of fixation, by diffusing fluids into the testis, result in disruption of the cytoplasm, especially in multinuclear cells. Methods of fixation were selected which preserved the latter in sufficient numbers to follow their development. It appears that the two secondary spermatocyte nuclei, produced after the first meiotic division of the primary spermatocyte, tend to remain together forming a binucleate cell, which becomes four-nuclear after the second meiotic division. These nuclei, now haploid, are capable of frequent multiplication within the cell before each, then designated a spermatid, eventually becomes transformed into a spermatozoon. The significance of post-meiotic multiplication is discussed.

Introduction

IN the past multinuclear cells have been noted in transverse sections of the seminiferous tubules of the fixed and stained fowl testis. Guyer (1909) stated that in maturation divisions there is a decided tendency for the nuclear phenomena to proceed independently of divisions of the surrounding cytoplasmic mass. Adamstone and Card (1934) implied that multinuclear cells were abnormal cell products caused by feeding on diets which were deficient in vitamin E, while Zlotnik (1947) regarded them as abnormal products of cell-division.

Multinuclear cells have demanded more attention since methods of studying unfixed tissue have been more widely used, as they are regular features of living cell preparations from the testis. In a preliminary report of a study of the living germ-cells from the testis of the fowl (Lake and Smiles, 1952) it was mentioned that many of the spermatid nuclei appeared in this form. Recently, Tillmann and others (1955) studied testis cells of a number of mammals as well as the fowl, and concluded that polynuclear spermatocytes were of normal occurrence in healthy testis tubules. Furthermore, they believed them capable of continued development. In view of all these findings, it was considered desirable to re-examine the structure of the entire epithelium of

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the seminiferous tubule of the fowl, first by making observations on living cell extracts with the phase-contrast microscope, and secondly by using the ordinary microscope on fixed and stained sections and stained smears of testis cell extracts. By so doing the apparent physical state of the cytoplasm of the various living cells could be studied and then suitable methods of fixation employed in an attempt to obtain stained sections in as lifelike a condition as possible. Artifacts, produced both by chemically fixing cells and pressing them alive between slides and cover-glasses, could be assessed and due allowance made in the microscopical observations.

Materials and Methods of Observation

Brown Leghorn cocks during the height of their reproductive season, i.e. from March to June, were selected for the examination of an active germinal epithelium.

By observing the change in the degree of Brownian movement of cell particles in grasshopper neuroblasts at various stages of the mitotic cycle, Carlson (1946) concluded that cells were in the least viscous state during metaphase. Viscosity was highest during the anaphase and early prophase and it lessened in later prophase. Lewis (1951) made similar observations on cells in tissue culture, and Ross (1954), using a more objective method of refractometry to measure changes in the water and solid content of the cytoplasm during cell-division, tended to confirm the phenomenon in grasshopper spermatocytes. From this it was considered reasonable to believe that the physical state of cytoplasm would be a contributory factor in determining their degree of preservation in a tissue subjected to chemical fixation. So, in the first part of the present study, a subjective estimation was made of the degree of concentration of dissolved matter in the cytoplasm of the various cells comprising the germinal epithelium of the cock. Hereafter this phenomenon will be referred to as the apparent cytoplasmic concentration. Living cell extracts were taken from the testis in the manner described by Lake and Smiles (1952), and placed on No. 1 cover-glasses. These were then placed on glass slides which were covered with a thin layer of agar that had been dissolved in 0·85% saline solution, and the apparent cytoplasmic concentration of cells was examined by phase-contrast microscopy. The general appearance and stability of the cytoplasm and the extent of movement of cell particles within it were noted as criteria in this judgement. Due consideration was given to the likely effects of cover-glass pressure in causing changes in physical state when interpreting the observations, since Strangeways and Canti (1927) and Marsland (1951) have drawn attention to the adverse effect that pressure has on the structure of cells. Each preparation was examined for a period of only 2 or 3 minutes, or until such time as cytoplasmic streaming or cell rupture became evident. Repeated observations showing a correlation between a certain division stage in a cell and a particular structure were considered sufficient justification for assuming that cover-glass pressure had not exerted any effects up to the time that estimates of apparent cytoplasmic concentration were made

As a guide to the differentiation of cell types in testis extracts preliminary observations were made on numerous stained sections of seminiferous tubules. In such preparations the different types were distinguished by the spatial relationships of their nuclei in the seminiferous tubule, and a comparison could be made with those seen in preparations of living cells. In the second part of this work, fixed and stained testis material was used. As a preliminary step, routine sections were made of tissue fixed in the following fluids: Susa, Bouin's, B 15, B 3, Worcester's, Carother's, Carnoy's, Carnoy-Lebrun's (McClung, 1950), and Orth's (Hotchkiss, 1948), and stained with Ehrlich's haematoxylin and eosin.

An examination of the sections showed that, regardless of the type of fixative used, the cytoplasm of all cells in the germinal epithelium cannot be fixed simultaneously, and the preservation of whole cells is not easily obtained. In view of these conditions it was decided to attempt as rapid fixation as possible of very small pieces of testes so that the entire cytoplasm of cells might be precipitated instantaneously. Three fixative fluids which contain fairly good cytoplasmic preservatives and which have a fairly high rate of penetration into tissues were chosen. These were Worcester's fluid, Bouin's fluid (see above), and Orth's fluid saturated with mercuric chloride, which is similar in composition to Helly's fluid. Cocks were killed by dislocation of the neck vertebrae and, within 5 minutes of death, the testes were removed, cut into fragments measuring about 3 cubic mm and placed in the solutions in 25-ml tubes. The fragments were left for 24 hours and then treated as usual in the post-fixation period, according to the constituents present in the fixatives. They were embedded in paraffin wax and sectioned at 5 μ for microscopical examination. All sections were stained with Ehrlich's haematoxylin and eosin.

As rapidity of fixation was thought to be an important factor in preserving germ-cells, it was decided, in addition, to extract living cells from the testis and fix and stain them on cover-glasses. A Pasteur pipette of very fine bore was dipped into a testis and a quantity of cells extracted and placed in a drop of either Bouin's fluid or the modified Orth's fluid. After half a minute the cells were smeared very gently on the surface of a cover-glass, another cover-glass being used as a spreader. The smear was dried, by wafting it in the air, and stained.

Results

The physical state of the cytoplasm of living germ-cells and the effect of certain chemical fixatives on the cytoplasms

Comparisons of observations on cell smears, testis sections, and living cells showed that in the sections the individual germ-cells, and the germinal epithelium of the seminiferous tubule, are not always easily preserved in bulk by histological fixation methods. From the examination of living cell preparations by phase-contrast microscopy several facts soon became evident. Most germ-cells in an active testis never appeared to possess the same apparent cytoplasmic concentration as the cells, for example, of squamous epithelium

of the mouth or ciliated epithelium from the vagina of the hen. Those in the dormant state, in early prophase or late anaphase and telophase had the majority of their mitochondria concentrated close to the nucleus in the Golgi zone. The Brownian movement of intracellular material was virtually nil. During late prophase, metaphase, and early anaphase, however, the cytoplasm became lighter in appearance and there was a scattering of cytoplasmic organellae. The general appearance was that of a decrease in cytoplasmic concentration with an increased Brownian movement of particulate material. Multinuclear cells were invariably present in living cell preparations.

All the seminiferous tubules in particular testis sections, fixed with any one of the three fixatives under special investigation in this work, did not show ideal preservation, but examination of a number of the best sections gave sufficient observations for the structure of the whole germinal epithelium and the individual cell types to be established. Estimates were made of the degree of preservation of testis cells which will be employed as a guide to the future development of a more suitable fowl testis fixative to preserve germ-cells in bulk. Worcester's fluid contains mercuric chloride and formaldehyde, which, when combined, rapidly penetrate and harden the cytoplasm of cells. Cells in the tubules near to the edges of testis fragments generally presented better pictures than those in the interior, and often in such positions Worcester's fluid proved useful in preserving those germ-cells with the more fluid cytoplasms. Fig. 1, A represents a field in the germinal epithelium where most spermatids have metamorphosed and the basal cell layers are actively

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Fig. 1 (plate). Unless otherwise stated, all cell preparations have been stained with Ehrlich's haematoxylin and eosin.

A, portion of a section of a tubule showing exhausted epithelium with a few remaining spermatids (spd) and developing spermatozoa (sp). The basal cells are beginning to multiply to regenerate the epithelium. Type 2 spermatogonia are visible in prophase (p) and in metaphase (m), for the production of primary spermatocytes. Dormant type 1 spermatogonia (d). Worcester's fixative.

B, section of a tubule showing disrupted binucleate cells (b); trinucleate cells (t) resulting from the separation of one nucleus and its collar of cytoplasm; primary spermatocytes in diplotene stage (dp) before production of binucleate cells; spermatid nuclei (spd) developing into those of spermatozoa. Worcester's fluid.

C, from a testis section showing binucleate cells (b) and trinucleate cells (t) after one nucleus has been disrupted and excluded. Worcester's fluid.

D, testis cell smear showing binucleate cells; one just formed from a primary spermatocyte division (br) and one in prophase of the second meiotic division (bp) to produce a four-nucleated cell. Four-nucleated cell (e) with each nucleus in the late prophase of a post-meiotic division. Multinuclear cell (f) formed by post-meiotic multiplication of haploid nuclei. Bouin's fluid.

E, binucleate cells in metaphase to produce four-nucleated cells. Tendency to disrupt is evident. From a testis section fixed in modified Orth's fluid and stained with methyl green and toluidine blue.

F, from a testis section showing two secondary spermatocyte nuclei in metaphase of the second meiotic division. This gives rise to the four-nucleated cell. Worcester's fluid.

G, testis cell smear showing spermatid nuclei developing into those of spermatozoa from the multinucleate condition. Bouin's fluid.

H, testis cell smear. Below, multinuclear cell containing numerous haploid nuclei before their metamorphosis into those of spermatozoa. Above, another such cell containing spermatid nuclei which are reduced in size and developing into those of spermatozoa. Bouin's fluid.

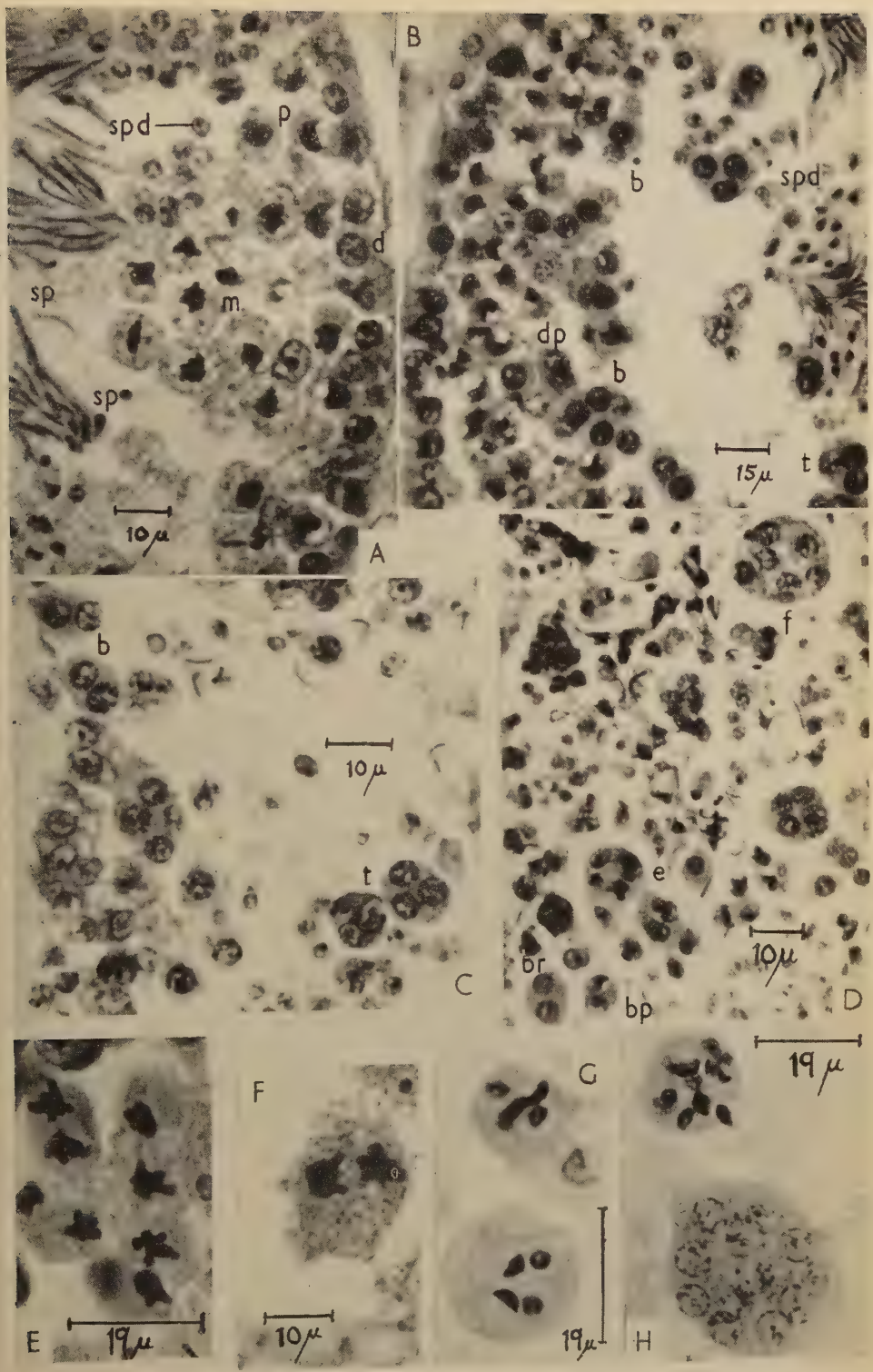
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FIG. 1

P. E. LAKE

regenerating the epithelium. Several whole cells in metaphase are to be seen. Modified Orth's fluid rapidly penetrated the small testis fragments and both whole cells and those in the metaphase stage of division were well preserved (fig. 1, E). This can be attributed to the presence of (*a*) potassium dichromate, which fixes protein material homogeneously without granular precipitation and preserves lipid-protein-polysaccharide complexes in cells, and (*b*) mercuric chloride which penetrates rapidly and hardens cytoplasm. Bouin's fluid, although proving excellent for the preparation of nuclear contents for subsequent staining, did not prove so efficacious as the other fluids in penetrating and fixing testis fragments to preserve cytoplasm. Possibly this is because the acetic acid it contains penetrates very rapidly and has a swelling action which is disruptive to germ-cells, while picric acid, its chief protein precipitant, has in contrast quite a slow rate of penetration (Medawar, 1941), and thus arrives in parts of the testis too long after the acetic acid to be of any benefit. On the other hand, it was useful as a fixative for cell smears, since under these conditions almost instantaneous penetration and fixation of cells by all constituents of the fluid occurs. Substituting modified Orth's fluid for fixation of smears produced better cytoplasmic preservation, but owing to its effect of precipitating fluid secretions of the testes the preparations were not suitable for photomicrography.

The cellular structure of the germinal epithelium and the appearance of multinuclear cells

The cellular structure of the germinal epithelium as seen in fixed and stained testis sections will now be described. Where necessary, it will be indicated how the implications from the above-mentioned observations bear on the cytological preservation of certain types of cells.

Before describing the individual cells of the epithelium, it may be useful to give a general impression of activity in the seminiferous tubules. From serial sections it can be seen that there is a constant regeneration of the basal cell layers (the spermatogonia and primary spermatocytes) as the spermatids metamorphose into spermatozoa towards the lumen. The entire activity may be likened to that in a layered, holocrine, secretory type of epithelium where in any one microscopical field basal cell types on the basement membrane can be in the resting state or in division, while cell layers towards the lumen are actively maturing (secondary spermatocyte stage) or shedding products (spermatid and spermatozoon stages). In different sections of the tubule, and sometimes also in the same transverse section, the epithelium shows different stages of cell activity depending on the differential maturation rates of the cells towards the lumina. Contrary to the conditions in some mammals no repetitive waves of identical cell activity appear to occur along the length of seminiferous tubules at regular intervals (Roosen-Runge and Giesel, 1950; Cleland, 1951). There are no such waves in man (Roosen-Runge and Barlow, 1953).

Observations on transverse sections of seminiferous tubules of testes fixed

in modified Orth's fluid show that absolutely clear lumina are not often visible in an active fowl testis. Instead, secretions from Sertoli cells, and cytoplasm which has sloughed from around the developing spermatozoan heads are prominent. Also it would appear that if secondary spermatocytes and spermatids become loosened from the germinal epithelium, they can move down the lumina of tubules and continue development at the same time. Only when the spermatozoa are free or completing development, and basal spermatogonial divisions are prominent in the regeneration of a particular area of the epithelium, is there sometimes a clear lumen (fig. 1, A).

The nuclei of the Sertoli cells, which are the most difficult to locate, have been adequately described elsewhere (Zlotnik, 1947). One observation should be stressed, however: namely, that these cells are not just localized in certain parts of the germinal epithelium; their cytoplasms form an extensive ramification between the germ-cells, thus making intimate contact with them. From electron microscope studies of the rat testis, a Sertoli cell syncytium has been suggested (Watson, 1952).

The mode of division of spermatogonia and their role in the production of primary spermatocytes is interesting as it appears that one daughter-cell from each division reverts to a resting stage to continue to perpetuate the cell race by later activity. For descriptive purposes this may be termed a type 1 spermatogonium. By further division the other daughter-cell, type 2 spermatogonium, gives rise to two primary spermatocytes, and in a tubule section is often seen in a field where the epithelium is in a regenerative state as spermatozoa are completing development. Fig. 1, A shows several type 2 spermatogonia in late prophase and metaphase stages of division. In the prophase they are clearly defined single cells with a small nucleo-cytoplasmic ratio. Their cytoplasm is more easily preserved in fixed and stained sections than that of the subsequent spermatocyte cells, which have a more rapid rate of division and apparently a less concentrated cytoplasm.

Early primary spermatocyte cells in the leptotene stage are distinguishable in tubule sections from type 2 spermatogonia, since there is an increase in the nucleo-cytoplasmic ratio, and they are frequently found associated with a more obvious layer of resting type 1 spermatogonia. In the late leptotene stage the nucleo-cytoplasmic ratio is greatest, and a few, together with those in zygotene and pachytene stages, appear isolated and wrapped up in Sertoli cell cytoplasm. Their cytoplasm tends to be obscured and thus they give the impression of being multinucleate cells. Guyer (1909) considered these as true multinuclear cells, but it is in the next series of events in the process of the maturation of male gametes that true multinuclear cells seem to arise. Fig. 1, B shows a region of the tubule epithelium where primary spermatocyte nuclei are in the diplotene stage.

When the first meiotic division has occurred, the two nuclei seem to remain together in a common cytoplasm, and a binucleate cell is thus constituted. Fig. 1, C, D shows several such cells. These and subsequent divisions of the nuclei are the most rapid in the testis, and in living cell preparations the

anaphases are seldom seen. In spite of this, however, sufficient pictures were seen in smears, sections, and living cell preparations to suggest that the following train of events occurs in the production of spermatozoa. The two secondary spermatocyte nuclei when formed soon divide within the same common cytoplasm to give four haploid nuclei (fig. 1, D); metaphase stages in the course of their production are shown in fig. 1, E and F. The four nuclei can then either metamorphose directly into four spermatozoa (fig. 1, G), or, as more often occurs, undergo a phase of post-meiotic multiplication, to produce more haploid nuclei (fig. 1, D and H) and thus eventually a greater number of spermatozoa, each with the same genotype. Such cells are not easily preserved in their entirety by histological fixation. There is no real telophase stage, and consequently from observations on living cells it is possible that the cytoplasm is never very concentrated between divisions. Phase-contrast microscopy of the living cells has shown that there is an area of cytoplasm immediately surrounding each nucleus where the cytoplasm is most concentrated, and in histological preparations this phenomenon is marked by a tendency for a heavier precipitation of the cytoplasm in this region. Strangeways and Canti (1927) reported a similar effect when they studied the changes occurring in living cells after the introduction of various fixative fluids, especially osmium tetroxide, saturated picric acid, or 10% neutral formalin. As a result of this constitution in the cytoplasm, in cell preparations of the testis and more especially in fixed and stained sections, separating nuclei and cytoplasms can frequently be seen in multinuclear cells, and trinuclear cells result (fig. 1, B and C). This occurrence, together with the general difficulty of preserving the cytoplasm of germ-cells in the fowl testis, leads to the scarcity of observable multinuclear cells in fixed testis material prepared for sectioning.

Before the haploid nuclei begin to transform into the nuclei of spermatozoa they undergo a reduction in size; the chromatin appears to form large aggregations within them. Spermatid nuclei of multinuclear cells before metamorphosis into spermatozoa are seen in fig. 1, H, and some undergoing metamorphosis in fig. 1, G and H. During the course of metamorphosis the developing spermatozoa gradually separate, surrounded by a cover of cytoplasm (fig. 1, B). The final stages of sperm formation consist of the elongation of the spermatid nucleus to form the head and the shedding of most of the cytoplasm. Their liberation has been best observed by Lake and Smiles (1952); it was further mentioned by Lake (1954). The facts that all the nuclei of multinuclear cells are more or less synchronized in division and that each final spermatid nucleus appears to transform into that of a spermatozoon indicate that the multinucleate condition is not an abortive procedure. The almost mature spermatozoa do not seem to be directed, as well-defined groups, towards any particular cell in the germinal epithelium, as has been generally implied in the past by workers who consider that Sertoli cells have some kind of attraction for mature spermatozoa.

Discussion

Little need be said of the structure of individual cells of the germinal epithelium, as adequate factual descriptions of fowl testis cells can be found elsewhere in the literature (Miller, 1938; Zlotnik, 1947). However, some brief mention should be made of the mode of production of primary spermatocytes from spermatogonia. The multiplication of the latter involves the simultaneous perpetuation of their race and would appear to be allied to the procedure which has been described previously for the rat (Roosen-Runge and Giesel, 1950; Clermont and Leblond, 1953) and for the monkey (Clermont, 1953). From the present study it appears that one daughter-cell of a spermatogonial division remains as a spermatogonium on separation, while the other further divides to give two primary spermatocyte cells which are then ready to begin meiotic division in the process of the maturation of male gametes.

In the majority of testis sections, one cannot fail to be impressed by the lack of definition of whole cells in the germinal epithelium. From the results of the present work this is understandable since the epithelium is in a very dynamic state. The action of fixative fluids, containing components with differing diffusion rates, is one of disruption on the cells with apparent low cytoplasmic concentrations. Thus, if the object of an investigation is to study whole cells in the seminiferous tubule in sections prepared in the conventional way, it is important to realize that for fixation there is a critical maximum size of tissue fragment for best preservation. It has been shown that even when quite small pieces of testis are placed in fixative fluids, the edges of subsequent sections taken from them always reveal the best-preserved entire cells. Part of the inability to observe whole cells in the germinal epithelium, treated by the usually prescribed methods, is due to the fact that the fixatives have been devised by those workers only interested in the preservation of chromosomes in germ-cell nuclei or in some specific cytoplasmic organellae. Cytological fixatives penetrate into tissues by the process of diffusion (Medawar, 1941), and it may be suggested that the slow diffusion of the constituents of fixative fluids, themselves diffusing at different rates into the testis and having differing effects on the preservation of cell cytoplasm, is likely to cause disruption in cells, especially if the latter have cytoplasm which tends to be in a fluid state. An ideal cytoplasmic fixative, like osmium tetroxide, generally has the slowest penetration rate into tissue and its beneficial action on tissue are generally antagonized by the more rapid penetrators in the fixative fluid, which are also the most destructive to the cytoplasm.

In contrast to fixed and stained sections, living cell preparations from fully functional testes show an abundance of multinuclear cells. It is considered that this discrepancy is accounted for by the difficulties of fixing actively dividing cells of the germinal epithelium in bulk, for the reasons mentioned above. Phase-contrast microscopy shows that on the whole the apparent cytoplasmic concentration of such cells tends to be lower than that found in those which form the static epithelia of the tracts and ducts of many bod

organs. The latter are easily preserved in bulk in fixed and stained sections, but germ-cells are not clearly defined in sections from active testes. An extreme example of the more fluid state of a cell correlated with poor definition in stained sections can be seen during the metaphase stage of division; the cytoplasm stains very lightly owing to the dispersed state of cytoplasmic material. By phase-contrast microscopy cells in the early prophase and late anaphase stages of cell-division are observed to be those with the most concentrated cytoplasms and this phenomenon corresponds with a high stain retention when they are appropriately fixed and stained. Since it has been observed that the multinuclear cells are those which are involved in the most rapid division phases, presumably a more fluid state would exist in their general cytoplasm for a relatively long period of the division phase. This condition, together with the existence of a more concentrated zone immediately surrounding each nucleus, would create areas of unequal density in the cytoplasm. It is suggested that such a system could easily disrupt when the testis is prepared for microscopical examination by histological fixation methods. Autolysis, pressure changes, and differential cytoplasmic precipitation effects, to which cells are subjected as the components of fixative fluids diffuse into the testis, are the likely causes of such disruption. Support for this argument is provided by the observations that when the fixative fluids employed are of a suitable composition to render them highly penetrative, and are used in such a manner as to cause as rapid and instantaneous a fixation as possible, multinuclear cells are preserved in greatest numbers. It is significant that previously when germ-cells from vertebrate testes had been subjected to rapid chemical fixation, multinuclear cells were observed in such numbers as to warrant particular attention. Guyer (1909) described their appearance in stained smears of fowl testis cells, and Stroganova (1952) described them in smears taken from the testes of the rat, mouse, and rabbit. Some mention should be made of the fact that the appearance of multinuclear cells has been commonly described as a particular manifestation of degenerative changes in the testis tubules of mammals and birds. From the results of the present work this would be expected, since during periods of testicular inactivity the cells of the germinal epithelium would have more concentrated cytoplasms and thus would be more easily fixed in bulk, and readily observable in the sloughing epithelium. In some cases the so-called 'giant cells' in abnormal testis tubules (Mason, 1926; Gordon and others, 1943; Nakanura and Makino, 1950; Maqsood, 1951; Schwartz and others, 1951; Kano, 1952) are clearly due to a coalescence of abnormal cytoplasms of different types of cells in the sloughing germinal epithelium, but this phenomenon in no way contradicts the present deductions on multinuclear germ-cells in normal testes.

It is considered that sufficient evidence has been given to show that post-meiotic multiplication of spermatid nuclei occurs in the fowl testis. This phenomenon has been described previously during a study of spermatogenesis in the body louse, *Pediculus corporis* (Hindle and Pontecorvo, 1942),

and in the fruit fly, *Drosophila melanogaster* (Pontecorvo, 1944). That haploid nuclei of the bird testis are capable of mitotic divisions was suggested by the early observations of Blount (1909), when supernumerary spermatozoa were shown to be present and undergo several mitotic divisions in the periblast region of the developing pigeon embryo after one spermatozoon had formed the male pronucleus and fused with that of the egg to initiate embryonic development. The daughter-cells disappeared at a later stage of embryonic development.

Further and more precise quantitative studies are necessary to examine any seasonal fluctuations in the numbers of multinuclear cells, and also the full significance of the post-meiotic multiplication in the field of genetics. With respect to the latter, the following point deserves mention. It has been observed that in sections of the testis there exist some multinuclear cells containing few and others many spermatid nuclei. If this is not wholly accounted for by their being representatives of successive stages of division then it might indicate that some genotypes are replicated in larger numbers than others, and that when it occurred, the subsequent chances of fertilizing an ovum would be biased in favour of gametes of the former type. If each genotype is replicated in equal numbers, however, then the process of spermatid multiplication could be merely another means of ensuring that an abundance of sperm is produced for the successful completion of fertilization.

A final comment on the development of spermatozoa from multinuclear cells is that the phenomenon would seem to offer a more feasible explanation for the appearance of clusters of spermatozoa in stained transverse sections of testis tubules. The previous ideas which postulated some kind of chemotactic effect of Sertoli-cell cytoplasm, whereby sperm adhered and gained nourishment in groups, is difficult to comprehend, especially since the cytoplasms of the Sertoli cells appear to ramify extensively throughout the interstices of the entire germinal eipthelium. The suggested directional congregation of sperm would appear to be unnecessary.

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A Histochemical Study of the Adipose Cell of the Leech, *Glossiphonia complanata*

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With one plate (fig. 2)

Summary

The adipose cell of the leech *Glossiphonia complanata* has been studied both morphologically and histochemically. It is more or less globular in shape, with a diameter of from 10 μ to 40 μ; the protoplasm is clearly marked off into two zones, the ground cytoplasm around the periphery of the cell, and a denser, basiphil zone (termed the 'surround') which encloses the fat drops and the nucleus. Large thread-like mitochondria are scattered throughout the cell; they are especially numerous in the 'surround'.

Histochemical tests showed that the ground cytoplasm contained arginine, tyrosine, glycogen, RNA, and inorganic iron; the 'surround' has, in addition to these, much phospholipid, some unsaturated lipid, and some acid mucopolysaccharide.

The large fat drops and some other smaller fat droplets found in the ground cytoplasm are chiefly composed of neutral lipid, possibly triglyceride; they also contain some cholesterol or cholesteryl esters and some unsaturated lipid. The lipochondria of the ground cytoplasm and the 'surround' differ from these in that although they may contain some or all of the substances found in the large fat drops, their principal constituent is phospholipid. The mitochondria also react positively to the test for phospholipid.

Some granules found in the 'surround' and in the ground cytoplasm were shown to contain inorganic iron. The 'fuchsinophil' granules of Bobin are almost certainly identical with the lipochondria.

Both the large fat drop and the glycogen are reserve food stores.

Introduction

THOUGH the adipose cell is a very prominent feature of the connective tissue of rhynchobdellid leeches, it has received comparatively little attention. The first mention of this type of cell seems to be in a paper by Leydig (1849) quoted by Bobin (1950); later it was noticed by Bourne (1884), and also by Graf (1899), who, although recognizing its nature, did not study it in detail. Cytological investigations of the adipose cell in various leeches have been published by Scriban (1910, 1924), Abeloos (1925), and more recently by Bobin (1949, 1950). No histochemical studies of this cell have been made, with the exception of some that are noted in Bobin's 1949 paper, and incidental observations published in a paper by Cain (1947*c*). The present paper gives the results of a reasonably full histochemical analysis of the adipose cell of the leech *Glossiphonia complanata*.

Material and Methods

As mentioned in a previous paper, this species of leech is particularly suitable for this type of study: the cells of the body are large, the animal is

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abundant in fresh water and is easy to keep in the laboratory. The large size of the cells, making observation of the results much simpler and more certain, was most important; the only disadvantage of this material is the difficulty of getting out undamaged living cells. Because of this, most of the work has been with fixed material, studied in sections, though a few studies with the phase-contrast microscope have been possible. It is not proposed to give details of the histochemical tests in the paper itself; instead, the results, with some practical notes and references, are presented in summary form as an appendix (table 2).

Structure of the Adipose Cell

The adipose cells, distributed throughout the body parenchyma, vary greatly in size, from 10 μ or less up to 40 μ in diameter. Bobin (1950) has

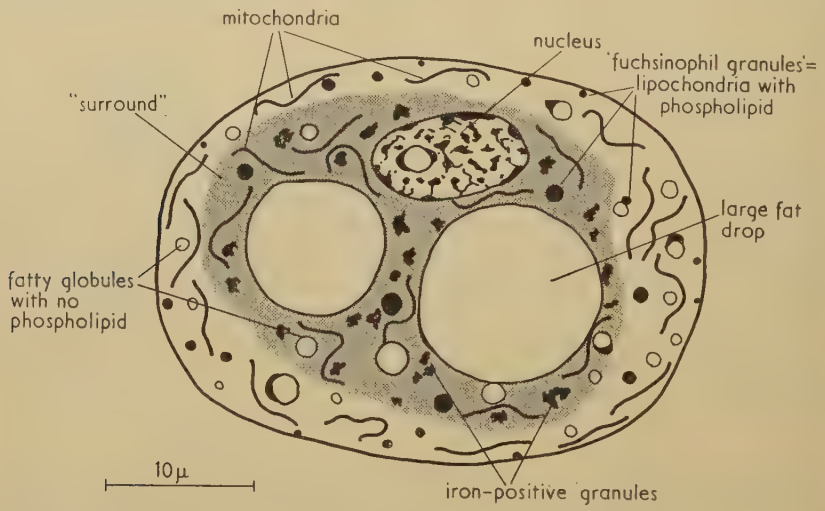

Fig. 1. Diagram of a generalized adipose cell of *Glossiphonia complanata*.

shown that in some cases the adipose cells can form pigment granules within themselves. The cell then becomes much elongated and may reach a length of 80 μ or more, with a diameter as seen in section of between 10 and 40 μ. The present description is only intended to refer to those adipose cells that have not formed this pigment. The main structural features of this cell are represented diagrammatically in fig. 1.

It is usual to find the largest of these cells deep in the parenchyma of the body, often close to the intestinal caeca. When a transverse section of *Glossiphonia* is examined, these cells are seen to be especially numerous in the region of the lateral coelomic sinus. Here their form is more or less globular; where the cells occur in between muscle-blocks or muscle-fibres, their shape is distorted to suit the available space. This point, noted by Bobin (1949), has been confirmed by the present observations.

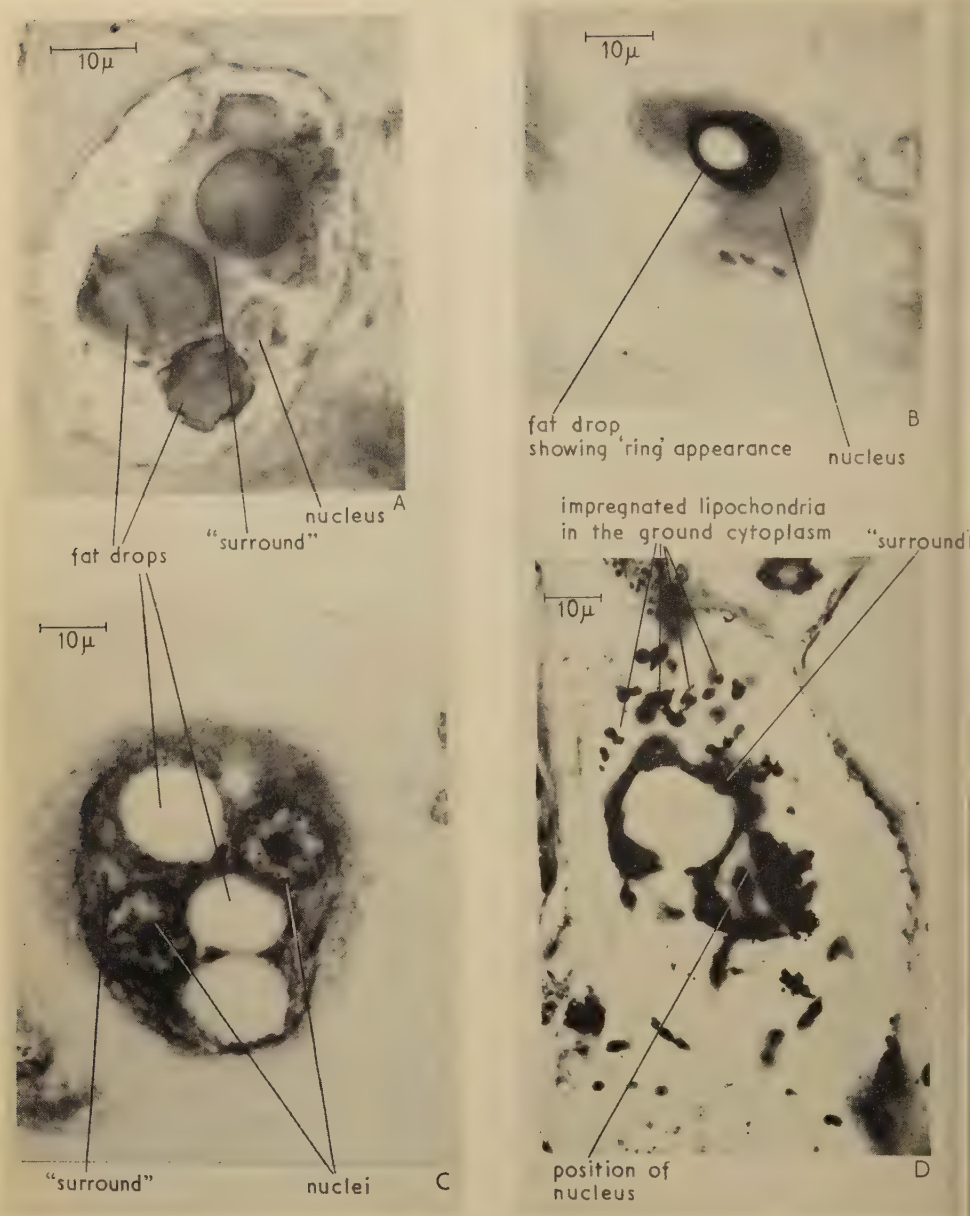

FIG. 2

S. BRADBURY

The most striking feature of the cell is the presence of very large fat droplets. In paraffin sections their position is indicated by well-defined vacuoles, but in gelatine sections the fat can be coloured by any of the usual fat-soluble colouring agents.

The protoplasm of the cell is clearly marked off into two zones, one around the periphery of the cell, the other forming the region immediately surrounding the fat drops and the nucleus. This latter zone of the cytoplasm is extremely basiphil, and it colours very easily with basic dye-lakes such as haemalum (fig. 2, A and B). Bobin terms this region the 'ergastoplasmic' zone, but as this term is now used in connexion with submicroscopic structure, it seems better to use a name that carries no structural implications. In this paper, the region of basiphil cytoplasm will be called the 'surround'. It does not appear to have a sharp boundary, but passes gradually into the ground cytoplasm; in places, strands of the more basiphil matter seem to pass right out towards the periphery of the cell. This distinction between the two zones of cytoplasm seems justified on the histochemical results. With phase contrast, the 'surround' appears darker than the ground cytoplasm and may therefore be assumed to consist of material of higher refractive index than the general cytoplasm.

The nucleus, which also occurs within the 'surround', is large, usually about 10 μ in diameter, and possesses a well-marked nucleolus. Often the adipose cells are seen to have more than one nucleus (fig. 2, B); Bobin (1949) has suggested that nuclear division may occur in these cells, preceding cytoplasmic division, but neither she nor I have in fact observed any mitotic figures.

Very large mitochondria are scattered throughout the cell, with a concentration in the 'surround'. They are thread-like, with a length of 10 μ and a constant diameter of about 1 μ. Mitochondrial techniques also show the presence of what Bobin terms 'fuchsinophil granules'. In her paper she does not put forward any suggestion as to the composition of these bodies. As a result of the present histochemical studies, it now seems possible to identify these fuchsinophil granules as lipochondria.

The Histochemistry of the Adipose Cell

Amino-acids and proteins

Because of the peculiar structure of the adipose cell, it was thought necessary to see if any particular region of the cell was rich in protein, and also to establish, if possible, the nature of the predominating amino-acids.

Fig. 2. The adipose cell of *Glossiphonia*.

A, coloured with Sudan IV. Three large fat drops are visible, with the nucleus and the 'surround'.

B, stained with Mayer's haemalum. Note the basiphil 'surround' and the two nuclei.

C, coloured with Sudan black. Note the 'ring' appearance of the large fat drop.

D, Aoyama's method. Note impregnation of the 'surround', and of some lipochondria in the ground cytoplasm.

Several methods were tried with success. One of the original tests is that due to Millon (1849), which depends on the presence in the protein molecule of the hydroxy-phenyl group. Although the reaction is given by any phenyl compound, except those doubly substituted in the ortho- and meta-positions, in the present tissue the only substance likely to cause a positive reaction is the amino-acid tyrosine. In the present work, a rationalized version of this test by Dr. J. R. Baker was used (Baker, 1956). The same author's version of the Sakaguchi test for arginine and other guanidine derivatives was also tried on the material. Both of these tests gave excellent results. As expected, the ground cytoplasm of the cell gave a positive reaction, but the 'surround' proved in both cases to contain greater concentrations of the substances than the ground cytoplasm. The nucleus reacted feebly to the test for tyrosine, but very strongly to that for arginine; negative results were obtained from the large fat drop; observations could not be made on any of the other cell constituents. It may be concluded that both tyrosine and arginine occur in the cell, though in much greater concentration in the 'surround' than in the general cytoplasm. The latter amino-acid is also represented in the nuclear substance.

A more recent test for proteins and their constituent amino-acids is the 'coupled tetrazonium' reaction (Danielli, 1947; Pearse, 1954). Diazonium salts, prepared by the action of nitrous acid in the cold on salts of primary amines, react in alkaline aqueous solution as diazonium hydroxides. These combine with the phenyl group of tyrosine, the indole group of tryptophane, and the imidazole group of histidine to give coloured products. Danielli introduced the procedure of intensifying the colour by using a bis-diazonium salt and then attaching a phenol to the free group of the protein-diazonium compound to give a strongly coloured compound. By the use of various blocking agents it is considered that the test may be made very specific, enabling the reacting amino-acid to be identified (Pearse, 1954, p. 56, table 5).

The coupled tetrazonium reaction was used on sections of *Glossiphonia*, the final coupling agent being 'H' acid (the sodium salt of 1·amino 8·napthol 3·6·disulphonic acid), which gives a reddish-purple colour as the final product. Blocking agents were used to treat some sections before applying the coupled tetrazonium reaction; they were benzoyl chloride, performic acid, and dinitrofluorobenzene. In each case, practical details were taken from Pearse (1954).

With the coupled tetrazonium technique, a very strong positive reaction was obtained from the 'surround', and less strong reactions from the ground cytoplasm and the nucleus. In the case of the 'surround', the reaction tended to be negative after treating with benzoyl chloride and dinitrofluorobenzene, but remained positive when the pretreatment was with performic acid. Though the blockage did not seem to be complete, it may be concluded that the constituent of the 'surround' responsible for the positive reaction is tyrosine, and that there is little histidine or tryptophane present in this region. The incomplete blockage may well be due to the fact that the technique is still very much in the experimental stage.

In no case was it possible to obtain positive reactions from the fat drop or from any other cell constituent, with the exception of the nucleus. It was thought at one stage of the investigation that lipid might be present in some form bound on to a protein framework, but these results do not support this possibility.

Carbohydrates

The basic technique for the demonstration of carbohydrates in tissue sections is the periodic acid / Schiff (PAS) reaction (McManus, 1946; Hotchkiss, 1948). The periodic acid acts on the C—C bonds in these substances; if this group is present as the 1·2·α-glycol configuration, CHOH—CHOH, it is converted into a form which will give a coloured product with Schiff's reagent. The equivalent amino- or alkylamino-derivatives of 1·2·α-glycol will also give compounds with an aldehyde type of structure on oxidation by periodic acid, and therefore they also will give a positive reaction with Schiff's reagent.

When the PAS reaction is applied to the adipose cell, a strong positive result is obtained in both ground cytoplasm and 'surround'. This may be due to the presence of one or more of the following groups of carbohydrates:

(1) polysaccharides, e.g. glycogen, starch;
(2) mucopolysaccharides, e.g. hyaluronic acid, chondroitin sulphate;
(3) muco- or glycoproteins, e.g. gastric mucoid, fractions of serum albumin and globulin;
(4) glycolipids.

Of the polysaccharides of group 1, only glycogen is known to occur in animal tissues. Because this substance is removable by the action of the enzyme diastase, it is relatively easy to identify. Slides were incubated in a solution of this enzyme at a temperature of 37° C for 1 hour before doing the PAS technique. Other slides were incubated for the same time in saliva, and in distilled water. It was found that incubation in saliva removed all the PAS-positive material from the ground cytoplasm of the cell, and to a very large extent from the 'surround'. Incubation in a pure diastase solution was not so effective as the saliva, but the action of the enzyme was apparent when compared with slides which had been incubated in distilled water before the test. From these results it may be concluded that glycogen is present in both zones of the cytoplasm of the adipose cell. As it may be supposed that this substance is acting as a reserve product, the same series of tests was carried out on sections cut from a leech which had been starved for 10 weeks before killing. In this case, the adipose cells were much smaller, and were almost entirely PAS-negative. From this experiment it seems justifiable to conclude that *Glossiphonia* stores some of its reserve food as glycogen.

As not all of the PAS-positive material was removed from the 'surround' by the action of saliva, it seemed likely that other groups of carbohydrates were present in this cell. In this connexion, relevant information may be

obtained from the metachromatic staining which results with some dyes, and also from the application of the methylene blue extinction test.

Though many dyes will produce metachromasy, only one (toluidine blue) was used in this study. It is generally agreed that two forms of metachromasy may be recognized, the violet beta form, and the gamma type which is red. This latter form of metachromasia is most often caused by sulphate esters, whilst polymerized carbohydrates or phosphate compounds give rise to the beta metachromasy. Nucleic acids, as reported by Wislocki, Bunting, and Dempsey (1947) and by Pearse (1954), may also cause this type of metachromasy.

It is certain that neutral mucopolysaccharides, though giving a very strong PAS reaction, do not show metachromasy. In the adipose cell of the leech, the 'surround' is often seen to show gamma metachromasy with toluidine blue. The chromatin is also found to give an appearance resembling metachromasia. These facts, together with the observation that the 'surround' shows a positive reaction to the PAS technique even after the action of diastase, seem to indicate the presence of an acid mucopolysaccharide. When the PAS reaction was carried out on material which had been fixed in a precipitant fixative such as Zenker's fluid, the positively reacting substance was very often noticed to be concentrated at one pole of the 'surround', near the fat globule. It was thought necessary to confirm that this diastase-fast substance was, in fact, responsible for the metachromasy. A slide was stained with toluidine blue in the usual way and mounted in distilled water, and a single adipose cell was carefully drawn, the position of the metachromatic material being noted. The same side was then placed in acid alcohol for 15 seconds to remove all the toluidine blue, and after washing in distilled water was carried through the usual PAS routine. The same cell as before was relocated and redrawn; this time the position of the PAS-positive material was noted. It was found that they coincided, so that the two reactions taken together seem to signify the occurrence in the cell of an acid mucopolysaccharide. This substance has not at the present time been further identified; it may prove to be hyaluronic acid or some heparin derivative. No evidence was available to indicate that the positive PAS reaction was due to any other polysaccharides apart from those of groups 1 and 2 already considered.

Nucleic acids

The Feulgen reaction and the usual control were done on paraffin sections of the animal. As expected, the only positive reaction was obtained from the nuclei, so that it may be said that in the adipose cell of *Glossiphonia* the desoxyribonucleic acid is restricted to the nuclear chromatin.

During routine morphological studies on this cell, involving staining with haematein lakes, basic fuchsin, and other basic dyes, it was noticed that the cytoplasm was very basiphil, especially in the region of the 'surround'. A measure of this basiphilia may be obtained by the use of the methylene blue extinction test. Originally due to Pischinger (1926, 1927) it has been modified

by Dempsey and Singer (1946). The capacity to bind methylene blue at very low pH is indicative of either sulphate groups, i.e. acid mucopolysaccharides, or nucleic acids (Pearse, 1954). In the adipose cell, the 'surround' is capable of binding methylene blue even at pH 2·6, a figure comparable with that of the nucleus. Although some of the cytoplasmic basiphilia could be due to the presence of acid mucopolysaccharide, it was thought more likely that ribonucleic acid (RNA) was responsible.

This was checked by the pyronin / methyl green staining method due to Jordan and Baker (1955). With this, the nucleus is a greenish colour, but the nucleolus and the ground cytoplasm together with the 'surround' are very strongly coloured with the pyronin. This is strong presumptive evidence that the compound causing the cytoplasmic basiphilia is ribonucleic acid, but it is not possible to say that this is definitely so until an enzymic digestion has been carried out on the material before staining. It was not possible to obtain crystalline ribonuclease, nor to prepare it as described by McDonald (1948). A technique was, however, developed for using treated human saliva as a source of this enzyme (Bradbury, in press); by incubating slides in this at a temperature of 60° C for 1 hour before staining with pyronin methyl green, the colouring of the cytoplasm and the nucleoli by pyronin was entirely prevented. Incubation of similar slides in distilled water was used to check that no cytoplasmic basiphilia was being removed by simple solution in the hot aqueous media. As pointed out in the paper on the method, a further test is necessary to see that all diastatic activity of the saliva has been removed by the process of inactivation; this was done by applying the PAS reaction to slides of liver which had been incubated in the treated saliva. If the inactivation has been complete, there should be no diminution in the intensity of the reaction given by the glycogen in the liver-cells. If these precautions are observed, the results are very reliable, and it becomes possible to use this, in conjunction with staining by pyronin / methyl green, for the localization of RNA.

In this particular cell, the ground cytoplasm possesses large amounts of RNA, but even more is concentrated in the 'surround'.

Lipids

For a preliminary histochemical study of the lipids, material fixed in neutral formaldehyde-saline was used. In the early stages of the work, when the fat was coloured with Sudan IV or with Sudan black to determine the location of the lipids, it was noticed that the large fat drop presented a most curious appearance, which was usually that of a crescent or a cup (figs. 2, c; 3). These forms were very variable, being especially marked if the fixation had been at all prolonged. Such an appearance would be given if some part of the lipid in the drop were solid at room temperature and therefore unable to colour with the standard techniques; accordingly the methods used for colouring the fat with the Sudan colouring agents were tried at 37° C and 60° C. In both cases there was no increase in colour, and the crescents and cups were found as before.

One of the studies of living cells showed that the large fat drop appeare perfectly spherical, so that these crescentic appearances were regarded a possible artifacts. Starke in 1895 showed that after fixation some constituent of fat droplets were not rendered insoluble and were able to be leached out b the action of aqueous solutions. If the fat drops were lying free, their surface became irregular and shrunken, but if the fat were embedded in some matrix as would be the case in the adipose cell of *Glossiphonia*, then the surface are

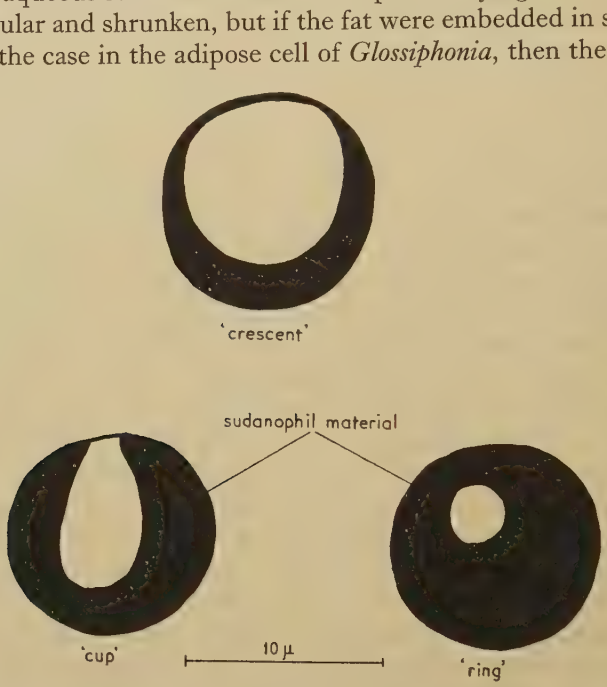

FIG. 3. Diagram showing the 'crescent', 'cup', and 'ring' appearances of the large fat dro resulting from inadequate fixation.

could not change, so that the material remaining would assume the form cups and crescents. In his paper, Starke figures some which are identical wit the appearances found in the present work.

In order to prevent the formation of these solution artifacts, the fixatio time was kept as short as possible. Baker (1946) and Cain (1947*b*) showed th calcium added to the fixative acts as an indifferent ion, tending to hold lipi in place. This was tried, with formaldehyde-calcium as the fixative and dichr mate-calcium as the postchroming fluid; the large fat drops then colour uniformly with Sudan IV or Sudan black. In addition, the latter reveale numerous lipochondria both in the 'surround' and in the ground cytoplasm It seems that the true condition of the large fat drop is that of a sphere, t crescent- and cup-shaped appearances resulting from solution of some of i substance during the preparation of the object.

This fat drop may attain a diameter of 18 μ, though a more usual size about 12 μ. In the smaller adipose cells it is usual to find only one, but in t larger cells found near the coelomic sinuses, as many as six fat drops occur m

in the 'surround'. Where several occur in one cell, they are usually smaller than when only one is present.

The coloration of the fat drop with the Sudan colouring agents indicates that the lipids of which it is composed are liquid at room temperature. Further tests were applied to try to discover the nature of these lipids. Cain (1947*b*) has established the mechanism of the coloration of lipids with Nile blue sulphate. Neutral lipids will take up from aqueous solutions of Nile blue only the oxazone and the free base, both of which are red. Acidic lipids, on the other hand, will take up the oxazone and combine with the free base to form blue compounds. Hence a distinction can be drawn between neutral and acidic lipids. With Nile blue, the large fat drop colours red, together with some lipochondria in the ground cytoplasm, whilst the 'surround' and other lipochondria often become blue. By the use of Sudan black, it can be shown that there is some lipid material in the 'surround', so that it may be concluded that the large fat drop and some other lipochondria contain neutral lipid, whilst the 'surround' and the remaining lipochondria are composed chiefly of acidic lipids. When tissues are fixed in Flemming's fluid, the large fat drop blackens, suggesting that it contains some unsaturated lipids. This blackening by osmium cannot be regarded as diagnostic of the presence of unsaturated C = C bonds, so two other tests were used. These were Ciaccio's technique, and the performic acid / Schiff reaction. The former depends on the fact that when lipids are postchromed, they become to a large degree resistant to extraction during paraffin embedding. According to Lison (1953) the time of postchroming required for this varies with the nature of the lipid, unsaturated phospholipids taking least (2–3 days), whilst others such as oleic acid need 7 days. Some, e.g. fatty acids and triglycerides, are not rendered insoluble even by a period of chromation exceeding 8 days. It is recognized that this technique does not fulfil all the requirements of a reliable histochemical test, but Kaufmann and Lehmann (1928) think it may yield some information of value regarding the presence or absence of unsaturated lipids. In this particular case, some of the material of the large fat drop was rendered insoluble after 2 days' postchroming, appearing in the sections as thin crescents or strands crossing the space formerly occupied by the lipid drop. This was noticed by Abeloos in 1925, when he wrote of 'sphères creuses' appearing with Ciaccio's technique. There is also a coloration of the 'surround' region after 2 days' postchroming. After 4 days' postchroming the appearance is the same, but after 6 days' much more material remains and the colouring of the fat drop is more intense. The appearance is now that of a very large crescent or a deep cup, exactly corresponding to the appearances resulting from faulty fixation. Evidently some constituent of the lipid drop is still being removed by the process of embedding. No further change was evident, even after the longest period of postchroming. All these results, taken together, could be explained by postulating the presence in the fat drop of some unsaturated lipid, some cholesterol or cholesteryl esters, and some fatty acid or triglyceride.

The performic acid / Schiff test gave a positive result both with the large

lipid drop and the 'surround', which agrees with the results obtained from th Ciaccio procedure. It thus seems justifiable to say that both the large fat dro and the 'surround' contain some unsaturated lipids. This unsaturation coul be caused by the presence of neutral fats, such as oleic acid, or by phospho lipids, both of which possess the ethylenic linkage in their molecule. Thi point was checked by the use of the acid haematein technique (Baker, 1946) The large fat drop was negative to this test; so the conclusion is that in thi case the unsaturation is due not to the presence of phospholipids, but t neutral lipids. A positive result was obtained with the acid haematein tech nique for other regions of the cell; these will be discussed later.

The presence of cholesterol or its esters was postulated as a possible ex planation of some of the results of Ciaccio's technique, so confirmation of thi was sought. Two tests are available, those of Windaus and Liebermann. Th former, for free cholesterol, was negative in this tissue. The latter gives positive reaction with both free cholesterol and its esters. In the modifie technique used, sections were placed in 2% aqueous iron alum at 37° C fo 24 hours in order to transform any cholesterol present into oxycholestero One batch of sections gave a negative result, but some other sections wer used which were positive to the test. On checking, it was found that thi second lot of sections had been stored in formol-calcium in the light for a fe weeks before applying the test. In Schultze's original version, sections wer exposed to full sunlight for several days in order to oxidize the cholesterol it seems likely that in this particular case, the quantity of cholesterol presen was so small that a 24-hour treatment with iron alum would not form enoug oxycholesterol to enable the characteristic greenish-blue colour to be forme with acids. After a long exposure to light, however, the amount of oxycholes terol formed was then sufficient to cause a positive result. The small amoun present may explain the negative result obtained with Windaus's metho so that it is not possible to say with certainty whether the substance is presen in the free state or, perhaps more probably, in the ester form.

Finally, it was supposed that the fat drop contained some substance whic was not rendered insoluble, even after 8 days' postchroming. This could b due to the presence of either fatty acids or triglycerides. The occurrence o fatty acids may be determined by the use of Fischler's technique, though, a Pearse (1954) points out, it is open to grave objections. As it is still the onl technique available for this group of compounds, it has to be used, thoug caution is needed in interpreting the results. When applied to the adipose cel totally negative results were obtained, and it is tentatively concluded tha fatty acids, as such, do not occur in the large fat drop. It seems that th presence of triglycerides must be inferred, on the grounds of exclusion of al other lipids. The above results can be explained by postulating the presenc in the fat drop of small amounts of cholesterol or its esters, and an unsaturate triglyceride. There is no phospholipid present in the large fat drop itself.

With the very sensitive Sudan black technique, many smaller lipochondri were noticed, some embedded in the 'surround', which was itself lipoida

thers scattered in the ground cytoplasm. They were very numerous, of sizes rom 1 μ up to about 12 μ or more in diameter. It was necessary to find out if hese were similar in composition to the large fat drop, and if they bore any elation to it. The lipochondria were found to give varying reactions to Nile lue, some being red, whilst others were definitely blueish, as was the 'surround'. This seemed to suggest that there were two series of lipochondria, ne resembling the large fat drop in containing chiefly neutral lipids, whilst he other was acidic in nature. The acid haematein test showed that the 'surround' was intensely positive, as were many of the lipochondria in the ground ytoplasm; this could account for the acidic nature of these bodies. In some ections, the acid haematein technique (colouring positive objects blue) was ollowed by the standard Sudan IV method. This procedure showed the 'surround' and some of the lipochondria in the ground cytoplasm as blue, but the arge fat drop and many other lipochondria were coloured red by the Sudan V. This seems to show conclusively that there are two chemically distinct ypes of lipid-containing bodies in this cell. One is composed chiefly of phospholipid, whilst the other contains only neutral lipids. Some of the former are een to be embedded in the 'surround', but quite a large proportion lie free in he cytoplasm. In these preparations it was noticed that very often there ended to be an association of phospholipid with the neutral lipid. The phospholipid may appear as a cap, or in the case of the large fat drop, as an entire oating to the other lipid. In some cases where the lipid globules are fairly arge, the phospholipid appears as small spherical masses applied to their urfaces (fig. 4). The possible significance of this will be considered later. In assing, it is worth noting that the acid haematein technique reveals the itochondria of the cell extremely well, on account of their large size and hospholipid content.

Some slides were prepared by the classical 'Golgi' techniques of Aoyama, nd Weigl's Mann-Kopsch method. It was found that the silver or osmium mpregnated the 'surround' very heavily, and also formed spheres in the round cytoplasm; there was no impregnation of the large fat drop (fig. 2, D). n view of the later histochemical results with the acid haematein and udan IV techniques, it was thought worth while to find out exactly what as being impregnated by the silver or osmium. This was attempted as ollows: some Aoyama preparations were made with a shortened period of ilvering, and with gelatine embedding instead of the usual paraffin. Frozen ections were cut, toned in gold chloride, and then subjected to the standard udan IV technique. It was found that the neutral lipids were still visible, oloured red, whilst the 'surround' was blackened, together with some bodies n the ground cytoplasm. The location of these in close proximity to the other ipids was exactly similar to the location of phospholipid as revealed by the cid haematein test. It seems certain that in this particular cell, the 'Golgi' echniques result in an impregnation of the sites of phospholipid. The similarity of these findings to those reported by Cain (1947*a*) for the gut-cells of he same animal is worth noting.

With mitochondrial techniques, such as that of Metzner, the acid fuchsi reveals, besides the mitochondria, the 'fuchsinophil granules' noted by Bobi (1949). The 'surround' is also very fuchsinophil. The nature of these fuch sinophil granules was not studied by Bobin, but as a result of the present wor it seemed possible that they were, in fact, the same lipochondria which cor tain phospholipid, and impregnate with silver and osmium. It was found tha there was a good correspondence in position of the fuchsinophil objects in th

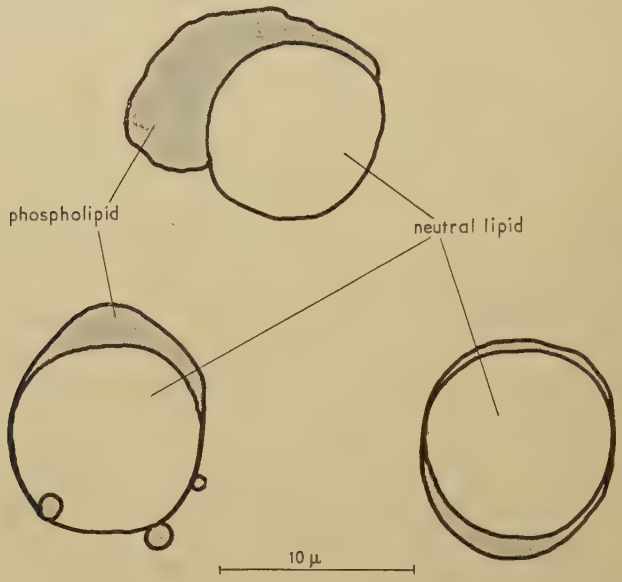

FIG. 4. Diagram to show association of phospholipid and neutral lipid.

cell, and the phospholipid. Similarly, it was found that there was a uniformi in the size ranges of the fuchsinophil granules and the lipochondria with th phospholipid. This was confirmed by measuring the diameters of both typ of lipochondria in cells coloured with acid haematein and Sudan IV. Th totals, recorded for 25 cell-sections, were expressed in the form of a histogra and compared with a similar series of measurements obtained from a Metzn preparation (fig. 5). The remarkable similarity between the size ranges of th fuchsinophil granules and the lipochondria which contain phospholipid, most striking, when contrasted with the wide scatter of the similar histogra for the neutral lipids. Statistical checks showed that there was no significa difference for the two sets of values obtained for the fuchsinophil granul and the phospholipid. Though it was not possible to perform the acid haem tein test and Metzner's technique on the same adipose cell, they were appli to consecutive sections in the series. Again the distribution of the phospholip and the fuchsinophil material was identical; in this case, as the acid haemate test was carried out on material embedded in paraffin, it would not norma be possible to say with certainty that the positive result indicated the presen

of phospholipid. As the distribution of the material positive to acid haematein is the same in both gelatine and paraffin sections, however, this reservation does not apply. It thus seems certain that the fuchsinophil granules of Bobin are the lipochondria that contain phospholipid.

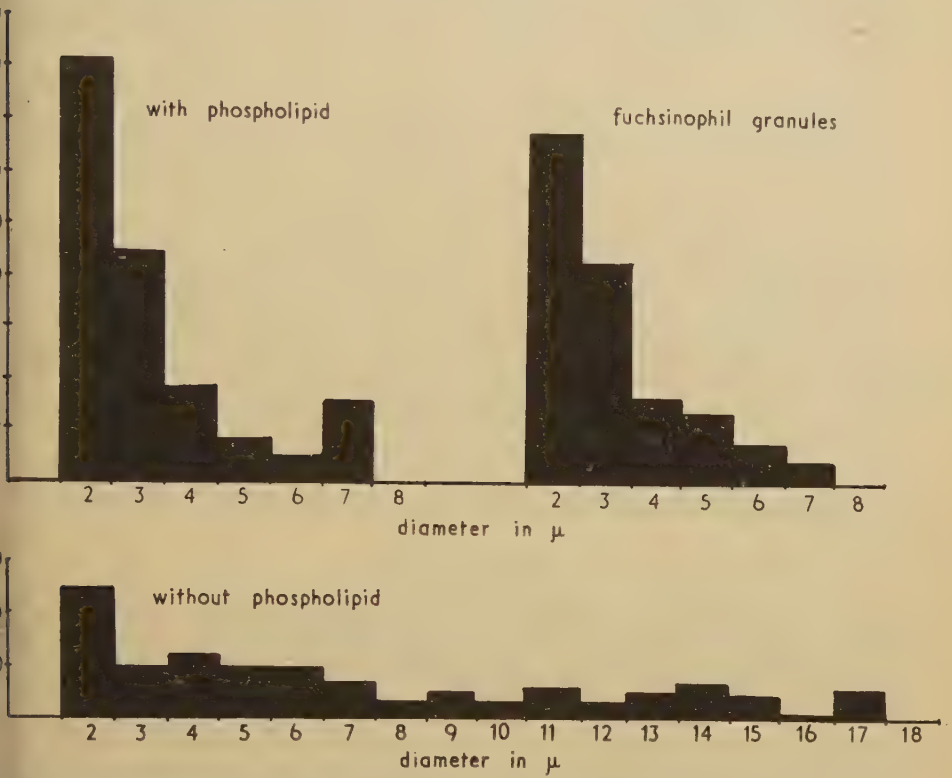

FIG. 5. Histogram comparing the size ranges of the fuchsinophil granules, and the two types of lipochondria.

The results of the histochemical analysis of the lipids of this cell may be summarized as follows.

There are two series of fatty globules. The first are composed chiefly of neutral lipids, which may be partially unsaturated, which may possess small quantities of cholesterol and its esters, and which may contain triglycerides. This series of globules ranges in size from 1 μ to 18 μ diameter. The large fat drop belongs to this category. The lipochondria in the second group differ from the others in that they contain large amounts of phospholipid. They have a smaller size range, 1–8 μ; the smaller ones are much more numerous than the large, and very often occur in association with neutral lipids. These lipochondria, together with the 'surround', impregnate with the classical 'Golgi' techniques, and represent the 'fuchsinophil granules' noted by Bobin. The 'surround' contains some unsaturated lipid; no trace of fatty acids could be detected in this cell.

Unclassified Remarks

During this work, the experiments on iron described in a previous paper (Bradbury, 1955) were repeated; the results were in agreement with the findings then reported. The inorganic iron is concentrated in the 'surround' and general cytoplasm of this cell, in many cases appearing as granular deposits.

These deposits were studied to see if they bore any relation to the other constituents of the cell, especially to the lipids, but as far as could be ascertained, they were scattered at random throughout the cytoplasm without any relationship to the other cell inclusions.

In view of the large concentrations of phospholipid in this cell, it was thought likely that there might be a strong concentration of phosphorylating enzymes present. Tests were carried out for the two main groups, i.e. alkaline and acid phosphatases. In both cases the results proved to be negative for the adipose cell, the only site of positive reaction being the brush border of the gut epithelium. This seems to suggest that if the phospholipid in the cell is acting in some way in the metabolism of the lipid components, then this does not take place through the agency of these phosphorylating enzymes.

The results of the histochemical work on this cell are summarized in table 1 (see appendix), and in the generalized diagram of the cell (fig. 1, p. 500).

Discussion

At this stage it is not possible to give a full account of the significance of the various compounds found to occur in this cell, but certain points may be touched upon. It was pointed out by Abeloos (1925) that the large fat drop was acting as a reserve store of food for the animal. He showed that in *Glossiphonia* starved for some months, there were no drops in the cytoplasm capable of blackening with osmium; a fact also true of young specimens. In the course of the present work, measurements were made of the diameter of this large fat drop. In animals which had been normally fed the mean diameter worked out at 10·5 μ, but in those which had been starved for 10 weeks this mean had become reduced to 4 μ. This represents a reduction in diameter of 62%, equivalent to a volume reduction of 94·5%. On further starvation the large fat drop may well be entirely metabolized. During starvation there does not seem to be any reduction in the quantity of phospholipid present in the cell.

The close association of phospholipid with the droplets of neutral lipid has already been noted, suggesting that there may be some connexion between the two; a most attractive hypothesis is that this phospholipid is the means by which lipid is passed into or out of the large fat drops. The absence of phosphatase in this cell seems to indicate that any such transfer is not mediated by phosphorylation mechanisms, but by other systems acting instead. There is some evidence from Ciaccio's technique that the layer of phospholipid immediately surrounding the large fat drop is of a greater degree of unsaturation than the rest. This point, commented upon by Abeloos, was also noticed in the

present study, and may be of significance in this process of transfer of material into and out of the large fat droplet. It is also of interest to note that not all of the reserve food store of the leech is present as neutral lipid. Some must be stored in this cell as glycogen, for a correlation has been found to exist between the glycogen content of the adipose cell and the state of nutrition of the animal.

Further investigation of the 'surround' region of this cell may well prove to be very interesting. Undoubtedly this part of the cell is extremely active in the metabolism, but at the moment the work has only reached the stage of elucidating, as far as possible, the normal chemical composition of the cell. Further speculation on functional aspects seems to be unwarranted.

I have great pleasure in acknowledging my debt to Dr. J. R. Baker, for supervising this work, and for stimulating and very helpful discussions, and to Prof. A. C. Hardy, F.R.S., in whose department the work was carried out. My thanks are also due to Mr. J. T. Y. Chou, for suggesting the possibility of using gelatine embedding in the modification of Aoyama's technique, and to Mr. M. H. Williamson for statistical advice.

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TABLE 1

Chief constituents of the various regions of the adipose cell

Region of cell, or inclusion	*Chief constituents*
1. Ground cytoplasm	Tyrosine, arginine, RNA, glycogen, inorganic iron
2. 'Surround'	Tyrosine, arginine, RNA, glycogen, phospholipid, some unsaturated lipid, some acid mucopolysaccharide, inorganic iron
3. Nucleus	DNA, RNA especially in the nucleolus, arginine
4. Large fat droplets	Neutral lipid, possibly unsaturated, some cholesterol or cholesteryl ester, some triglyceride
5. Lipochondria	Phospholipid, possibly some of the constituents of 4 above
6. Mitochondria	Phospholipid
7. Granules in 'surround'	Inorganic iron

TABLE 2

A summary of the histochemistry of the adipose cell of Glossiphonia complanata

Practical notes					*Results obtained for various regions of the adipose cell*						
Test or technique	*Fixation*	*Embedding medium*	*Thickness of section in μ*	*Reference*	*Ground cytoplasm*	*'Surround'*	*Nucleus*	*Large fat drop*	*Lipochondria*	*Granules in surround*	*Mitochondria*
Hg/nitrite	FS	C	15	Baker, 1956	++	+++	+	o	o	—	—
Sakaguchi	Z	P	8	Baker, 1947	++	+++	+++	o	o	—	—
Coupled tetrazonium	FS	P	8	Pearse, 1954	+	+++	+	o	o	—	—
C.T. after benzoylation	FS	P	8	Pearse, 1954	o	o	++	o	—	—	—
C.T. after performic acid	FS	P	8	Pearse, 1954	+	+++	+	o	—	—	—
C.T. after dinitrofluorobenzene	FS	P	8	Pearse, 1954	+?	o	+++	o	—	—	—
Feulgen	Z	P	8	Feulgen and Rossenbeck, 1924	o	o	++	o	o	—	—
Feulgen; no hydrolysis	Z	P	8	,,	o	o	o	o	o	—	—
Pyronin/methyl green	Z_3H	P	8	Jordan and Baker, 1955	pyronin ++	pyronin ++	MG +++	o	o	—	—
P/MG after ribonuclease	Z_3H	P	8	Bradbury, 1956 (in press)	o	o	MG +++	o	o	—	—
Periodic acid/Schiff	FS Z	P	8	Pearse, 1954	+++	+++	o	o	o	—	—
PAS after saliva digestion	FS Z	P	8	Pearse, 1954	o	+	o	o	o	—	—
Metachromasy	FS Z	P	8	Baker (unpublished)	o	++γ	++β	o	o	—	—
Methylene blue extinction	FS	P	8	Pearse, 1954	3·8–4	below 2·6	2·6	—	—	—	—
Sudan IV	FS FCa+PC FS+PC	G	10	Herxheimer, 1901	o	+	o	+++	+++	—	—
Sudan black	FS FCa+PC FS+PC	G	10	Baker, 1944, 1949	o	++	o	+++	+++	—	+

Nile blue	FS FCa FCa+PC	G	10	Cain, 1947*b*	o	blue	o	red	—	—	—
Acid haematein	FCa+PC	G	10	Baker, 1946	o	+++	o	o	some +++ some o	—	+++
Acid haematein: pyridine extraction	WB+PE	G	10	Baker, 1946	o	o	+++	o	o	—	o
Liebermann	FS	G	10	Lison, 1953	o	o	o	+++	—	—	—
Windaus	FS	G	10	Lison, 1953	o	o	o	o	—	—	—
Fischler	F	G	10	Pearse, 1954	o	o	o	o	—	—	—
Performic acid/Schiff	FCa FCa+PC	G	10	Pearse, 1951	o	+	+	+++	some +	—	—
Ciaccio	CF+PC	P	8	Lison, 1953	o	+	o	+ 2 days +++6 days	—	—	—
Aoyama	AF	P G	8 10	Aoyama, 1929	o	+++	o	o	+++	—	—
Mann-Kopsch	MF	P	8	Weigl, 1910	o	+++	o	o	+++	—	—
Metzner	ALT	P	2	Metzner and Krause, 1928	o	+++	o	o	+++	—	+++
Hirschler	ALT	P	2	Hirschler, 1927	o	+++	o	o	+++	—	+++
Perls	FS	P	8	Gomori, 1952	++	+++	o	o	—	+++	—
Gomori for alkaline phosphatase	ALC/AC	P	8	Gomori, 1952	o	o	o	—	—	—	—
G. for acid phosphatase	ALC/AC	P	8	Gomori, 1952	o	o	o	—	—	—	—

KEY: AF = Aoyama's fixative; ALC/AC = absolute alcohol / acetone mixture, ALT = Altmann's fluid; C = celloidin; CF = Ciaccio's fixative; F = neutral formaldehyde; FCa = Formaldehyde-calcium; FS = formaldehyde-saline; G = gelatine; MF = Mann's fluid; P = paraffin; +PC = with postchroming; WB+PE = weak Bouin followed by pyridine extraction; Z = Zenker's fluid; Z3H = Zenker fixation for only three hours; +++ = strong reaction; ++ = moderate reaction; + = weak reaction; — = no observation; o = negative.

Experimental Alteration of the Peri-renal Tissue of *Protopterus*

By D. E. MOORHOUSE

(*From the Department of Zoology, University Museum, Oxford*)

With two plates (figs. 2 and 3)

Summary

The experiments were carried out on *Protopterus aethiopicus* in East Africa. They were: the injection of commercial adrenocorticotrophic hormone, hypophysectomy, administration of ACTH after hypophysectomy, artificially induced aestivation, and artificially induced 'stress'.

The two elements of the lipid tissue show differing reactions to the experiments carried out. The large lipid cells appear to be under direct pituitary control: active secretion follows ACTH administration, hypophysectomy leads to a blocking of secretion. After 'stress' and hypophysectomy the small lipid cells develop sudanophil inclusions which are positive to the histochemical tests for steroids. This does not occur after ACTH administration.

The phagocytes of the endothelial system take part in the transfer of material within the peri-renal tissue; this is shown by their cytology after ACTH administration and 'stress'. Evidence from these experiments indicates that the round pigment cells characteristic of the normal animal are syncytial structures formed by the fusion of phagocytic cells containing pigment and remains of large lipid cells.

The steroid tissue shows little change in these experiments other than a decrease in the amount of steroid material after ACTH, and a more intense staining of the mitochondria after hypophysectomy.

The number of eosinophil leucocytes increases as the result of ACTH administration.

The round cell nodules showed no detectable changes in these experiments.

Introduction

In a previous paper (Holmes and Moorhouse, 1956) it was shown that there are strong indications of an evolutionary connexion between the peri-renal tissue of *Protopterus* and the cortical tissue of the adrenal glands of higher animals.

The present account is of an experimental investigation designed to discover whether the histologically distinct elements of the peri-renal tissue react to procedures known to alter the structure of the adrenal cortical cells of higher animals.

Material and Methods

The animal studied was *Protopterus aethiopicus*. The individuals were collected in the same manner as those used in the previous work. They were all between 130 and 250 mm in total length.

[Quarterly Journal of Microscopical Science, Vol. 97, part 4, pp. 519–534, 1956.]

The experiments carried out were:

I. the injection of commercial adrenocorticotrophic hormone (ACTH);
II. hypophysectomy;
III. the administration of ACTH to hypophysectomized animals;
IV. artificially induced aestivation;
V. artificially induced 'stress'.

The histological and histochemical methods applied to these animals were the same as those described in the previous paper. All the animals were killed by decapitation. The kidneys with the attached peri-renal tissue were divided into five pieces, each of which was fixed differently for the appropriate histological and histochemical techniques.

Results

I. Effects of the adrenocorticotrophic hormone (ACTH)

The adrenocorticotrophic hormone used was the product manufactured by Organon Laboratories Ltd., marketed under the trade name 'Cortrophin'.

For use, 1 ml of solvent (distilled water containing 0·05% phenol) is added to one ampoule of the powder. All made-up solutions were used within 24 hours.

The hormone was administered by injection into the dorsal musculature of the fish at a point approximately 3 cm posterior to the heart.

Each injection consisted of one dose as specified in table 1.

To exclude the possibility that the phenol present in the solvent acted as a 'stressing' agent, a control experiment was carried out with this solvent alone. In this experiment one animal received three injections of 0·25 ml of 0·05% phenol in distilled water at 24-hour intervals. It was killed 24 hours after the last injection. Histological examination showed that in this animal there were no changes departing from the range of variation seen in normal individuals.

Table 1

Animal no.	*Dose (mg)*	*No. of injections*	*Time interval between injections (hours)*	*Killed after further no. of hours*
710	5	10	24	24
719	10	3	24	24
720	5	1	..	1
721	5	1	..	3
718	5	1	..	6
717	5	1	..	24
716	5	1	..	48
722	5	1	..	72

Table 1 lists the animals used in this study, and shows the dosage given and other information.

The lipid tissue. ACTH causes a depletion of phospholipid in the peri-renal tissue. This is brought about through a decrease in the number of cells containing large droplets which react positively to the acid haematein test (the large lipid cells) (fig. 2, A). The response of these cells to ACTH is as follows.

One hour after an injection of 5 mg of ACTH it is found that the intracellular droplets in an abnormally large proportion of these cells are negative to acid haematein, so that more than usual of the cells contain a mixture of positively and negatively reacting droplets, and some produce a reaction intermediate between a true positive and a negative. After a single injection of 5 mg the number of cells whose inclusions give a positive reaction continues to decrease for the first 24 to 48 hours, but at no time is the lipid tissue entirely devoid of cells with positive inclusions, although individual sections from material taken 24 to 48 hours after an injection may show none. Thereafter the recovery phase sets in. By 72 hours the number of large lipid cells is approaching normal, but these cells are unusual in that many of them giving an acid haematein reaction contain less than the normal number of droplets. They vary from cells with clear cytoplasm and few droplets spaced far apart in it, to the form typical of the normal animal, in which the droplets are so tightly packed as to obscure all other details of the cellular structure. A few mitochondria are to be seen in some of those cells in which the droplets are less numerous.

Approximately 24 hours after administration of the hormone, a large number of cells are to be found undergoing mitosis. One category of cell which is shown to be dividing is that characterized by a rounded nucleus, little cytoplasm, and no distinct cytoplasmic inclusions. It was suggested in the previous paper that these may be the cells from which the lipid cells are normally formed; indeed, cells of the same shape and staining reactions but containing a few large lipid droplets are occasionally to be seen undergoing mitosis.

At the same time as the phospholipid is disappearing from the liposomes of the large lipid cells, many extracellular droplets which are strongly positive to the acid haematein test appear in the tissue. The number of these rises steeply in the first 12 hours after an injection, and thereafter declines unless the injection is repeated. The size of the extracellular droplets is less than that of the droplets within the cells. They stain intensely by the mitochondrial and Azan methods.

The cells from which the acid-haematein-positive material has disappeared degenerate: the cell membrane disintegrates and frees the haematein-negative vacuoles, and the nuclei become pycnotic.

The results given by this series of experiments in which the animals received a single injection were supplemented by two experiments in which repeated injections were given (animals 710 and 719, table 1). These two animals provide confirmation of the evidence for cellular degeneration following the phospholipid release in that many pycnotic nuclei are to be found scattered amongst the small lipid cells, and in the existence free in the tissue of groups of haematein-negative bodies with the appearance of vacuoles. Some

of these 'vacuoles' retain their ability to stain by the mitochondrial and acid haematein methods.

The effect of ACTH administration on the small lipid cells is never to cause depletion of their content of fine droplets, nor any change in their staining re-

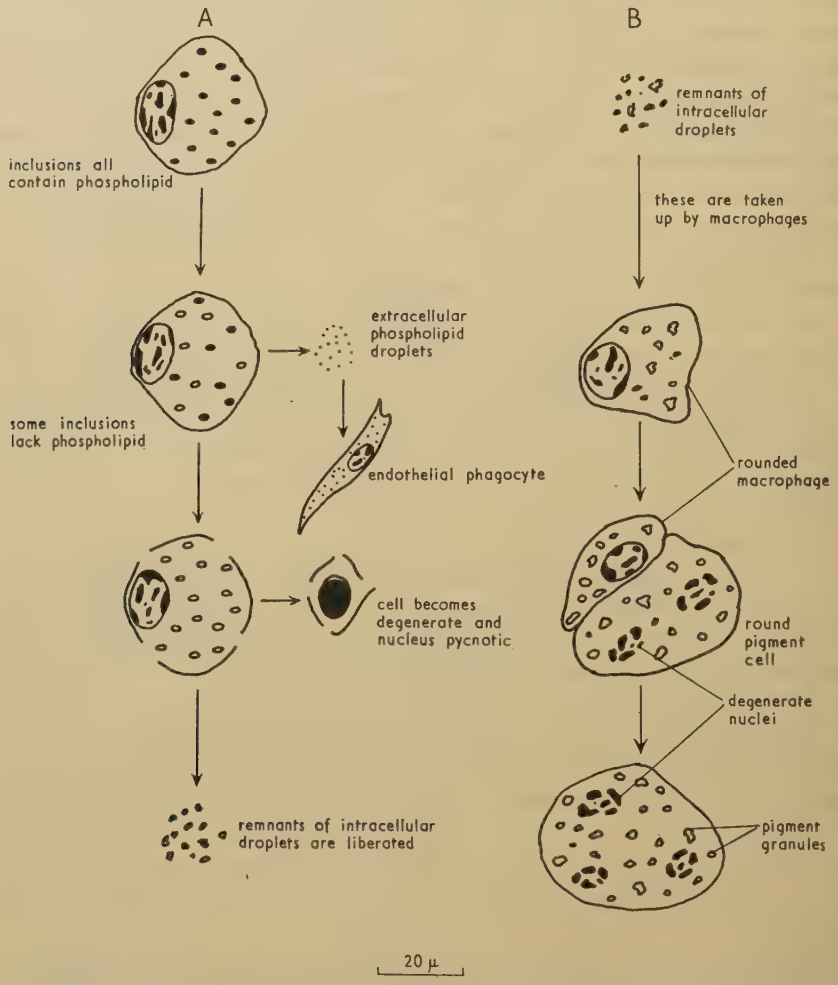


FIG. 1. Diagrammatic representation of A, the fate of the large lipid cells; B, ingestion of the breakdown products by phagocytic cells, and formation of round pigment cells.

action, but cells of this type form a substantial part of those which are found in mitosis after injection of the hormone. Mitosis in this type of cell is seen earlier than in the parental form of the large lipid cell. The number of cells seen in mitosis reaches a maximum 24 hours after an injection and is still high 72 hours after a single injection. Repeated injections do not increase the number of mitoses. It is interesting to find that these cells are capable of division when filled with their full complement of cytoplasmic inclusions.

Mitotic figures are so extremely rare in the normal material that it was not found practicable to establish a baseline figure of normal frequency of mitosis with which the experimental animals could be compared.

At the same time as mitosis is occurring in the small lipid cells there is an increase in the number of eosinophil leucocytes within the blood-vessels of the peri-renal tissue. They are to be found packed in the most minute capillaries. Many of them are so situated that it is difficult to decide whether they are in the blood-vessels or free amongst the peri-renal cells.

The endothelial phagocytes and round pigment cells. Three hours after an injection of ACTH the elongated endothelial phagocytes present a normal appearance, but thereafter some of them become filled with Metzner- and acid-haematein-positive droplets, of about the same size as the extra-cellular phospholipid droplets previously mentioned as appearing amongst the lipid cells (fig. 1). The rise in the number of droplets contained in the phagocytes is accompanied by a fall in the number of droplets lying free in the tissue. Occasionally the phagocytes may also contain pigment granules.

The round form of endothelial phagocyte reacts by taking up the haematein-negative 'vacuoles' which are liberated by the break-up of the large lipid cells, and also those less completely changed groups of inclusions which still retain their phospholipid staining reaction. The round phagocytes clump together in groups of four or five, the cell-walls degenerate, and the whole group becomes syncytial. Those droplets from the large lipid cells which retain their specific staining capacity are visible within these syncytial structures, which are most conspicuous for their high content of yellow or brown pigment. The nuclei of the individual phagocytes of which the syncytium is composed tend to break up, and can be seen as diffuse blocks or strands of chromatin, within the round pigment 'cell'. They never become pycnotic after the manner of the nuclei of the large lipid cells. Additional round phagocytes can be incorporated into pre-existing round pigment cells, appearing as an area containing relatively few pigment granules and a degenerate nucleus within one of the pigment cells containing otherwise very large amounts of pigment. This is also shown by the fact that occasionally groups of droplets containing material which stains by the mitochondrial and acid haematein techniques are to be found within the round pigment masses. It seems likely that this series of changes seen after ACTH administration is the usual way in which the dense round pigment 'cells' are formed in the normal animal, but that it is speeded up by the hormone. The hormone is therefore increasing the phagocytic activity of the macrophage system, which must be connected with the life-cycle of the lipid cells and their secretory activity, with lipid transfer and pigment formation.

The dendritic melanophores. There appears to be no change in number, distribution, or structure of the dendritic type of melanophore in any of the experimental animals.

The steroid tissue. Injection of ACTH causes a decrease in the amount of steroid material. So far as it is possible to determine, the number of the cells is not affected, the reduction taking place in the number of steroid-positive

spheroids within the cells. The amount of depletion varies between the individual cells in sections taken at different levels. Within a single injected animal various conditions of the steroid cells are found, ranging from cells packed with spheroids as in the normal, to ones with far fewer than the normal number. This depletion is obvious 6 hours after a single injection.

In the animals to which repeated injections were given, some of those steroid cells which contained the normal complement of spheroids were seen to contain also rods (possibly crystals) of sudanophil material. These were the only animals in which these objects were found in the peri-renal tissue. Crystals of steroid material are common amongst the sudanophil material in the adrenals of higher animals (Hoerr, 1936; Cain and Harrison, 1950).

There is no evidence of a recovery phase in the steroid tissue within 72 hours after injection.

The round-cell nodules. No consistent changes were found in this tissue in any of the experimental animals, and no indications which throw light on its nature and function.

II. Hypophysectomy

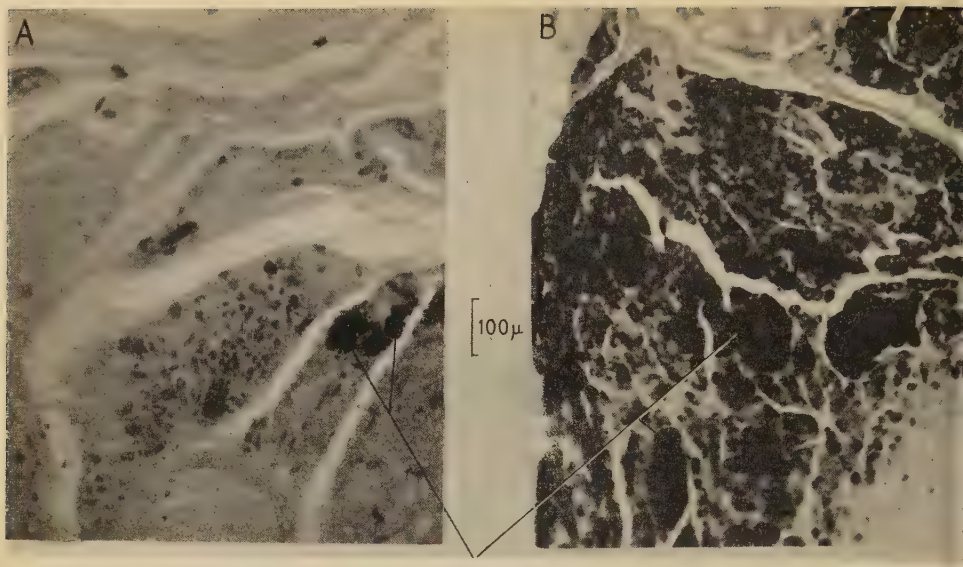
Preliminary dissections of these young animals, in which the skull is only partially calcified, showed that the position of the pituitary can be located by eye; and thus it was possible to plot its position accurately for use during the operation.

The method of hypophysectomy used consisted in passing a flattened needle, approximately one-tenth inch in width, along the median line between the two parasphenoids; if this was inserted slowly, whilst twisting, it was possible to drill a hole along the line of suture of the two cartilages into the cavity in which the pituitary lies. By this means the anterior lobe of the pituitary could be chopped up; the anterior lobe is large and distinct from the rest of the gland. Some of the tissue could be removed, but some remained in position: it had to be assumed that the remaining portions were so damaged and devascularized as to become non-functional.

The brains of all the hypophysectomized animals were removed after the animals were killed. They were fixed, serially sectioned at 10 μ, and stained by the Azan technique in order to survey the degree of damage inflicted on the pituitary. As a result of this examination a number of animals were not used any further when it was found histologically that substantial undamaged portions of the anterior lobe remained. All those to which reference will be made were animals in which the hypophysectomy was found to be successful (though it is still possible that some active nests of cells were undamaged and left in position when the brain was dissected out for histological examination).

Anaesthesia was induced by injecting 0·1 ml of 'Nembutal' into the dorsal musculature of the animal, and thereafter maintained with ether vapour.

Protopterus withstands hypophysectomy very well: it continues to feed, and appears to remain in good physical condition. After the operation the animal blanches, usually within a few hours (but occasionally this may take up to 24



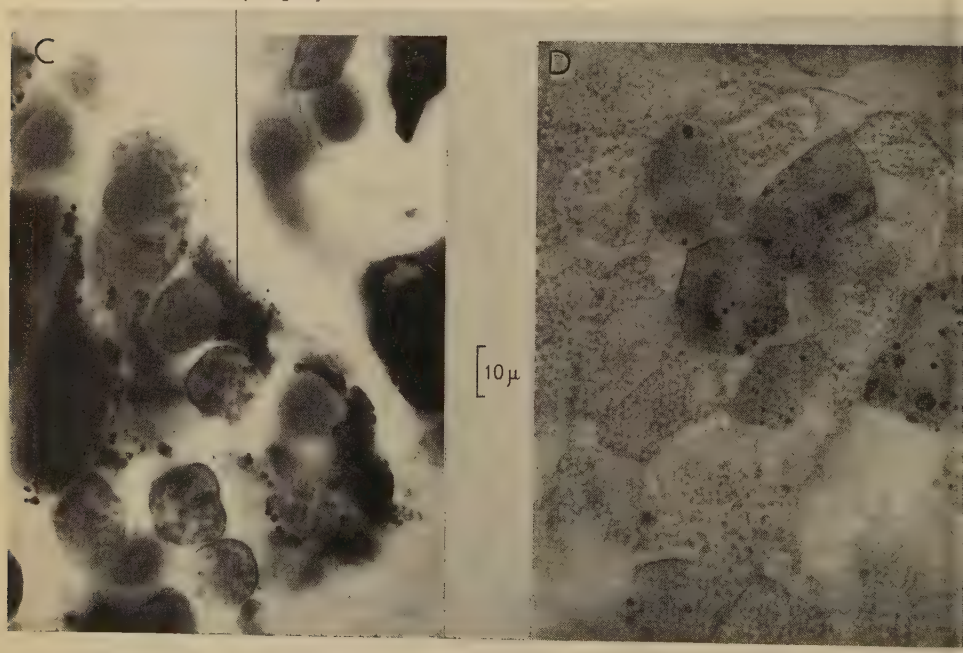


FIG. 2

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hours); it remains in this blanched condition for a week or so, and then slowly reverts to its normal dark colour.

The lipid tissue. The most striking changes take place in the lipid tissue.

Naked-eye examination of the kidney, immediately after dissection of the animal, shows a markedly altered appearance, in that the kidney is seen to be surrounded by a white 'sheath', sufficiently thick almost to obscure the pigment cells, which in the normal animal gives to the kidney a characteristic speckled appearance. It will be shown that this change is due to a thickening of the peri-renal tissue, due predominantly to an increase of the phospholipid content (fig. 2, B).

This increase in the quantity of phospholipid in the peri-renal tissue is due, in part, to an increase in the number of large lipid cells and also to an increase in size of the liposomes. The increase occurs in the main in the peripheral lipid cells which in the normal animal are arranged in a 'fasciculate' manner.

In addition to the many large lipid cells in which the droplets are acid-haematein-positive, there is intermingled with them a larger population than normal of cells with negative droplets. There is no evidence that these cells are breaking down after the manner of similar cells in the ACTH-injected animals, but a small number of pycnotic nuclei are to be found, along with occasional pigment granules, pigment masses, and extracellular haematein-positive liposomes, which suggest that a similar type of mechanism is at work dealing with degenerating cells as was found in the animals treated with ACTH. But it seems that in hypophysectomized animals the process of degeneration of the cells which have discharged their phospholipid content proceeds at a much slower rate than in the normal or ACTH-treated animal. There is some increase in the frequency of mitosis in the 'parental' type of cell without inclusions, but in none of the animals examined does it reach the same proportions as is found in an animal 24 hours after an injection of ACTH.

In the second group of lipid cells (those characterized by the presence of small cytoplasmic inclusions and a lobed nucleus) two differences are to be found when they are compared with the same cell type in the normal animal.

First, in many of them some of the granules are larger than normal. Secondly, they may contain 'spheroid complexes' made visible by an intense coloration with Sudan black.

The degree of increase in size of granules varies between the cells and often within the individual cell. The granules are never as large as the droplets or

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FIG. 2 (plate). A, section through lipid tissue. Acid haematein. ACTH injection (animal 710). Few haematein-positive lipid cells: most of the apparently positive cells are in fact erythrocytes.

B, section through lipid tissue. Acid haematein. Hypophysectomy, killed after 55 days.

C, section through lipid tissue. Metzner's method. ACTH injection (animal 717). Endothelial phagocyte filled with Metzner-positive droplets.

D, section through lipid tissue. Ashbel-Seligman reaction for steroids. Hypophysectomy, killed after 55 days. Steroid-containing spheroids in small lipid cells.

vacuoles found in the large lipid cells. The 'spheroids' are conspicuous in Sudan preparations since the cytoplasm and other inclusions are uncoloured or only feebly coloured by the Sudan (fig. 2, D). Often they are as much as 6 μ in diameter.

They appear in various forms, but in all cases the internum is an unstained sphere; the investing body (the sudanophil element) may be smooth and continuous, or broken up into separate granules. An irregular investing body tends to be more common in the cells which contain fewer spheroids and more of the normal granules of small diameter whose sudanophilia is feeble. The smooth, rounded spheroids are found principally in the cells whose granules are larger and wholly negative to Sudan.

The sudanophil material of the spheroids is dissolved by lipid solvents. It is not preserved by dichromate mordanting, and is thus invisible in mitochondrial preparations, in which the other cytoplasmic inclusions are intensely stained, irrespectively of their size.

Sudanophil spheroids were present in approximately 40% of the small lipid cells in one particular animal killed 55 days after hypophysectomy, but in this respect there was much individual variation between the individuals. They were, however, present to a greater or less extent in all the animals examined.

The sudanophil element of the spheroids reacts positively to the Ashbel-Seligman and phenylhydrazine 'steroid' tests, so that hypophysectomized animals are strikingly different from normals in that these reactions are not confined to the steroid tissue.

The endothelial phagocytes and round pigment cells. There appears to be a marked reduction in the total number of endothelial cells in the hypophysectomized animals. The dendritic type of endothelial phagocyte contains very many fewer than normal of the granules which stain by the mitochondrial method, or none at all (fig. 3, C). The ones which still have these inclusions tend to be grouped together; the others without positive granules often contain fine unstained inclusions which are a feature of these cells as found in the normal animal.

The rounded endothelial phagocytes are similar to the normal, though they contain less pigment. The pigment that is present is usually found in masses within these cells; individual granules of pigment are very scarce.

The only departure from the normal in the round pigment cells is the appearance that the number of pigment granules is greater, and it is very rarely that one sees evidence of the incorporation of pigment-containing phagocytes into the existing syncytia.

The steroid tissue. The mitochondria in the steroid cells stain more intensely with acid haematein and with Metzner's method. There is no evidence of an alteration in the number of cells giving a positive reaction to the 'steroid' tests, but the concentration of steroid material within the cells increases; often it appears not in the form of 'spheroid complexes' but as a homogeneous material in the cytoplasm.

III. The administration of ACTH to hypophysectomized animals

Two animals which had been hypophysectomized 94 days previously were injected four times, at 24-hour intervals, with 5 mg of ACTH in 0·25 ml of solvent. They were killed on the 98th day, and the peri-renal tissue fixed for examination. The brains also were studied histologically, and the sections showed that the anterior lobe of the pituitary was almost entirely destroyed.

The lipid tissue. The kidneys of the animals showed the 'white sheath' appearance as seen after hypophysectomy alone. Histochemical examination showed that this was due to the presence of abnormally large amounts of phospholipid. But the distribution of the phospholipid in the large lipid cells was conspicuously different, for, although in the acid haematein preparations some of the large lipid cells were unchanged from the condition seen after hypophysectomy, that is, they were packed with intensely positive droplets slightly larger than those found in the normal animal, in others the haematein-positive material was greatly changed in form and arrangement. The effect of the hormone was to cause a clumping of the droplets within the cytoplasm and a partial or complete loss of the individuality of the separate inclusions (fig. 3, A). Groups of droplets were partially fused together forming a mulberry-shaped object, or more completely fused to form spheres, which might be up to 10 μ in diameter. Often the spheres have additional droplets attached to them at their periphery. Occasionally the spheres are not homogeneously black with haematein, but instead have a colourless internum and a black externum, as a complete or partially complete ring. They are thus like large 'spheroid complexes'. And many of the large lipid cells have a vacuolated appearance, with small positive droplets lying in the mesh which forms the walls of the unstained vacuoles.

There is also a good deal of extracellular haematein-positive material lying in various parts of the peri-renal tissue, in the form either of homogeneous droplets or spheroid complexes. The latter have such very various shapes that this name is perhaps inappropriate for them. They may be hemispherical, or saucer-shaped, and smaller particles of positive material may be attached to them. Their shape is endlessly variable, and there are indications that the material of which they are composed may not be homogeneous, for in Metzner preparations a single spheroid may show local variations in the intensity of its positive reaction to the stain, the externum being strongly positive, feebly so, or almost negative at different points. These variations are paralleled in the haematein preparations, but the variations are less striking in the thick frozen sections than in the paraffin sections used for the mitochondrial technique. The size of these extracellular bodies varies widely, reaching a maximum of 10 μ.

The second element of the lipid tissue, the small lipid cells, may contain sudanophil spheroids. But compared with the hypophysectomized animals the spheroids are very much rarer and their externum is never so thick or so

intensely sudanophil. They continue to give a positive reaction to the 'steroid tests and are removed by lipid solvents.

The number of cells undergoing mitosis is markedly reduced from the stat found in both the ACTH and hypophysectomized groups.

There is an increase in the number of granular leucocytes, as in the norma animal treated with the hormone.

The endothelial phagocytes and round pigment cells. The general picture i similar to that in the normal animal receiving ACTH.

Many phagocytes of the dendritic type are filled with Metzner-positiv granules; others, as in the hypophysectomized animal, are devoid of thes structures; often they contain pigment granules. The rounded type contai much pigment, both in the form of separate granules and pigment masses Also they may contain large pale brown spheroids, apparently derived fro the extracellular spheroids previously mentioned in this group of animals some of them stain to a greater or less degree in Metzner preparations. I addition to the pigment many of these cells contain acid-haematein- an Metzner-positive droplets of the same size as those found in the large lipi cells.

The round pigment cells appear to be essentially the same as in the norma animal receiving ACTH. And this material provides further evidence that th rounded phagocytes can be incorporated into already-existing round pigmen cells; for in these animals many of the round pigment cells contain groups c acid-haematein- and Metzner-positive droplets, along with the spheroids pre viously mentioned, whose shape and mitochondrial staining reaction are s distinctive.

The steroid tissue. The mitochondria of the steroid cells stain more intensel than normal by the acid haematein and Metzner methods.

The concentration of steroid material within each cell is, however, muc lower than in the hypophysectomized animal. As in the normal, and norma treated with ACTH, the steroid material is in the form of 'spheroid com plexes'; it is not homogeneously distributed in the cytoplasm as after hypo physectomy alone. There is no evidence that the number of steroid-containin cells has changed.

IV. Artificially induced aestivation

Most of the knowledge of the phenomenon of aestivation has been gaine from *P. annectens*. It is a response to the drying out of the swamps. Observa tions have shown that the fishes can survive encysted for up to 9 months of th year, during which time the ground is perfectly dry and hard.

Despite prolonged search in the mudflats on the margins of Lakes Victori and George, no aestivating animals were found. It is probable that aestivatio is not a normal phenomenon in the life of the *P. aethiopicus* living in these tw lakes. There is only slight seasonal variation in the level of the lakes in norma years, and no seasonal drying out of swamp areas such as occurs in the Gambi where naturally aestivating specimens are to be found rather easily.

An attempt was therefore made to induce the animals to aestivate artificially.

Debbies (kerosene containers), from which the tops had been removed, were filled with mud from the lake margin. The mud was allowed to settle, and was topped up to give a 12-inch thickness of mud with 3 inches of clear water above. When filled, the debbies were placed in the open air, sheltered from the direct rays of the sun. Two fish were introduced into each debbie, and the surplus water was allowed to evaporate. In approximately a week the water evaporated and the mud consolidated. The debbies were then taken inside the building and stored in the relatively cool aquarium room. 'Earth lids' were noticed in a number of the debbies.

In the first experiment, lasting 55 days, only one animal survived out of a total of 12. When examined before killing it was found to be properly encysted, with cocoon membrane and a properly developed 'breathing hole'.

After this failure further attempts to induce the animals to aestivate were made with mud from different localities and with markedly different consistencies; also the rate of evaporation was controlled. In spite of very many attempts, aestivation was successfully induced in only three cases.

Johnels and Svensson (1954) state that the cocoon membrane in *P. annectens* is pigmented, usually brownish-red. The membrane found around these specimens of *P. aethiopicus* was transparent, and as colourless as cellophane. This confirms the impression gained by Johnels and Svensson from photographs of *P. aethiopicus* cocoons published by Smith (1939).

No changes have been seen in the number, distribution, cytological structure, or histochemical reactions of the large lipid cells.

Some of the small lipid cells contain sudanophil spheroids. In general structure these are similar to those previously described in the other experimental groups, having an unstained internum and a sudanophil externum. However, the externum is never so thick nor so intense in its staining reactions as in those previously mentioned. The spheroids give positive reactions to the 'steroid' tests and are removed by lipid solvents.

Throughout the peri-renal tissue fine extra-cellular sudanophil droplets are to be found. They lie between the cells and are aggregated in large numbers along the edge of strands of connective tissue and the outer walls of blood-vessels.

The most obvious change in the aestivating animal is in the pigment found in the peri-renal tissue, and in the presence of pigment in the circulating blood. Numerous pigment granules are to be found scattered between the cells of the lipid tissue. Some of the granules, many more than in the normal, are within the cytoplasm of both the dendritic and the rounded endothelial phagocytes, but the majority are undoubtedly extracellular and there is much pigment within the blood-vessels.

A second change is in the intravascular granulocytes: the majority are degenerate and have lost their normally distinct cytological features. These changes are best seen in Leishman-stained blood-smears. Many of the cells have a pale watery appearance. Often the granules appear to have fused to form irregular

bands within the cell, which stain a dirty orange-brown colour. The nucleus becomes pale and almost homogeneous; often it appears to be breaking up into a number of blocks (fig. 3, D). In some cases the formation of a brown pigment within the cell accompanies the degenerative process.

In general these observations confirm those of Jordan and Speidel (1931). But the eosinophil destruction to which they refer seems to take place within the blood-vessels, and has not been seen elsewhere in the peri-renal tissue.

There is no evidence of any change in the remaining elements of the tissue.

V. Artificially induced 'stress'

In recent years many studies have been made of the effects of 'stress' on the cells of the mammalian adrenal cortex. Three of the more commonly used 'stressing' agents have been applied to *Protopterus*. These are adrenalin, formaldehyde, and low temperatures.

The adrenalin was used as a 1:1000 solution of the hydrochloride in distilled water. The dose was 0·1 ml of this solution injected into the dorsal musculature 3 cm posterior to the base of the skull. Three animals received 2; three, 4; and two, 8 doses, given at hourly intervals. Each was killed half an hour after the last injection.

Formaldehyde was used in a 0·4% solution in water (i.e. 1 volume of commercial formalin with 99 volumes of distilled water). The injections were once again into the dorsal musculature. Two animals received 0·4 ml of the solution and were killed 2 hours later; two received 0·5 ml and were killed after 4 hours; and two 0·5 ml, being killed after 24 hours. The only available cold chamber was a refrigerator at 8° C. Two animals survived for $2\frac{1}{2}$ hours at this temperature and were then killed.

The lipid tissue. After the induction of 'stress' there is a decrease in the number of large lipid cells giving a positive reaction to the acid haematein test; this is accompanied by an increase in the proportion of the cells whose inclusions are negative, and of others which contain a mixture of positive and negative inclusions. Many of the cells show a foamy, vacuolated appearance with small haematein-positive droplets, lying amongst unstained vacuoles (fig. 3, B). This is a rapid response, being evident in one animal $1\frac{1}{2}$ hours after the injection of adrenalin (the animals receiving two injections).

Three and a half hours after the initiation of 'stress' (the animal receiving four injections) the phospholipid depletion is well-marked: few large lipid cells giving a haematein-positive reaction are to be found in the tissue. These changes are accompanied by the appearance of fine extracellular phospholipid droplets. In a few cells there is evidence of the fusion of the haematein-

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FIG. 3 (plate). A, section through lipid tissue. Acid haematein. Hypophysectomy followed by ACTH. Vacuolated large lipid cells are seen.

B, section through lipid tissue. Acid haematein. Adrenalin stress.

C, endothelial phagocytes in lipid tissue. Metzner's method for mitochondria. Hypophysectomy, killed after 55 days.

D, blood-smear. Leishman. Hibernating animal. Degenerating granulocyte.

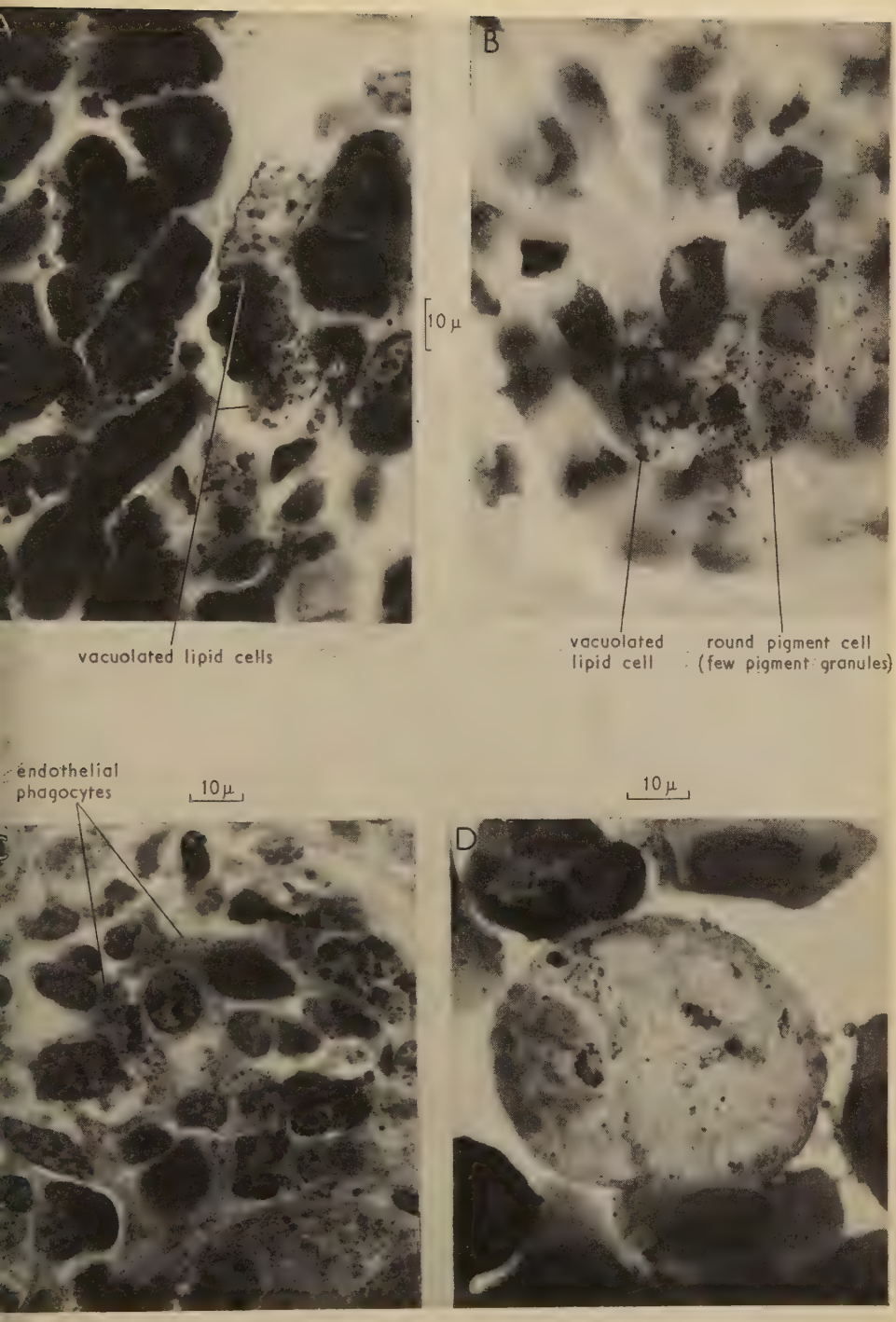

FIG. 3

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positive droplets to form larger spheres. A few large extra-cellular haematein-positive droplets are to be found within the lipid tissue.

Depletion of phospholipid is obvious 4 hours after the administration of 0·5 ml of 0·4% formaldehyde. After 24 hours few typical large lipid cells are to be found in the tissue, but a few are to be found which contain both haematein-positive and negative droplets. There are cells which contain few large droplets spaced apart in the cytoplasm, and which are identical in appearance with the cells found in the recovery phase following the administration of ACTH. The number of cells undergoing mitosis is small, but appears to be above normal. The frequency of mitosis is not sufficiently high to suggest that the cells resembling those of the ACTH recovery phase are being formed by cell-division as is the case after administration of the hormone.

Cold 'stress' brings about the same kind of changes in the large lipid cells.

After the induction of 'stress' spheroid complexes appear in the second element of the lipid tissue, the small lipid cells. As with the spheroids found in the previous groups of experimental animals, they are sudanophil, give positive reactions to the various 'steroid' tests, and are removed by lipid solvents, and often some of their other inclusions are larger than in the normal small lipid cells. Spheroid complexes are to be found $3\frac{1}{2}$ hours after the initiation of adrenalin 'stress' (i.e. after four injections). They do not appear until 24 hours after the administration of 0·5 ml of 0·4% formaldehyde. After $2\frac{1}{2}$ hours' cold stress only a few of the small lipid cells contain spheroids.

Endothelial phagocytes and round pigment cells. Two hours after the administration of 0·4% formaldehyde the majority of the dendritic endothelial phagocytes have their normal complement of Metzner- and haematein-positive granules. A few, however, are abnormally packed with granules. Four hours after formaldehyde injection the majority of the dendritic phagocytes are packed with the fine granules. Twenty-four hours after an injection they have returned to their normal state. At this time the rounded endothelial cells are seen to contain greatly increased quantities of pigment, generally in the form of masses, which appear to have arisen by the partial fusion of the individual granules. In a few cases separate pigment granules are to be found within the cells. A number of them contain blocks of sudanophil material. The round pigment cells are normal in appearance; occasionally they may contain lipid droplets.

The steroid tissue. There is little evidence of change in the steroid tissue as the result of 'stress'. In a few of the animals the concentration of steroid substances is below normal in some, but never in all the cells. In the cells which show this decrease the spheroids giving a positive reaction to the steroid tests are located on the outer perimeter of the steroid cells.

In some animals the mitochondria seem to stain more intensely after the induction of 'stress', but this appearance is so variable in different animals that it is impossible to make any generalization on the subject.

Discussion

The injection of ACTH has been shown to produce the following effects on the rat adrenal (Bergner and Deane, 1948; Yoffey and Baxter, 1949). There is a sharp decline in the amount of sudanophil and steroid-positive material in the first 24 hours; the depletion appears first in the reticularis, according to Yoffey and Baxter, and these authors consider that it is relatively slight in the fasciculata. Bergner and Deane, however, say that in 24 hours the sudanophil material disappears entirely from the fasciculata; the layer becomes widened, and 'watery vacuoles' develop at its outer surface. It is clear that, while details of the process are variable, ACTH administration may be said to cause a loss of sudanophil steroid-positive material in the adrenal cortex.

Hypophysectomy causes marked changes in the intracellular lipid of the cortex, and ultimately an atrophy of the tissue. In the mouse and guinea-pig there is a great loss of sudanophil material (Schweizer and Long, 1950; Chester Jones and Roby, 1954); in the rat the sudanophil material aggregates into droplets much larger than the normal, formed by clumping (Reiss and others, quoted by Bachmann, 1954). Schweizer and Long tried the effect of restoring the pituitary hormone to a hypophysectomized animal by implantation of a foreign pituitary. They found that the graft partially maintains the content of sudanophil material but does not wholly arrest the atrophic changes.

Dosne and Dalton (1940) used 4% formaldehyde and cold as stressing agents. With sudan colouring agents and osmium tetroxide they showed loss of lipids and hypertrophy of the cortex, followed by reappearance of the droplets. Later the lipids again decreased, if stress was continued. This was the 'exhaustion phase' of Selye's adaptation syndrome. They agreed with Selye's suggestion that decrease in lipid droplets signified an increased production of the adrenal cortical hormones.

Of the elements of the lungfish peri-renal tissue it is the steroid zone which has most obviously something in common with the mammalian adrenal cortex. If the conclusion of Cain and Harrison (1950) is accepted, the presence of sudanophil material in spheroid form is indicative of active secretion, and the reduction in the number of spheroids after ACTH administration shows that the secretory product is being discharged. The more intense staining of the mitochondria of these cells in hypophysectomized animals is undoubtedly significant, but the question of the connexion between the mitochondria of the adrenal cells and their lipid inclusions is one that has been much debated but never solved (see Hoerr, 1936).

Unlike the steroid cells in the lungfish, which appear to be relatively 'inactive', there is definite evidence of mitotic division, growth, and discharge of the phospholipid content of the large lipid cells, this being especially apparent during hyperactivity of the gland—after the injection of ACTH or the induction of 'stress'. In both these cases fine extra-cellular droplets containing phospholipid are to be found. At the same time there is evidence of breakdown and dissolution of the large lipid cells, accompanied by a recovery phase

with division of cells of a parental type, and the formation of phospholipid droplets within them.

Hypophysectomy appears to lead to a blocking of the normal cycle of secretion of the large lipid cells, not only in preventing the release of their phospholipid content but also of the breakdown of those cells which have lost their phospholipid content. Eventually there is some evidence of atrophy of some of the lipid cells, as is found in the mammal, but this is never so marked in the lungfish within the period of these experiments.

Hypophysectomy followed by the administration of ACTH leads to a release of the 'blocked' phospholipid of these cells, and is accompanied by the appearance within the tissue of large amounts of extra-cellular phospholipid and degeneration products of the large lipid cells.

The small lipid cells form a substantial part of the cells found in mitosis following hyperactivity of the gland. After 'stress' and hypophysectomy, many of these cells are found to contain sudanophil 'spheroid complexes'; they are never found after the injection of ACTH; it may be that like those of the zona glomerulosa, these cells are not directly under the influence of the anterior lobe of the pituitary, the appearance of sudanophil spheroids after hypophysectomy being due to the general metabolic upset of the animal. If this is so the question remains as to why the number of small lipid cells undergoing mitosis should increase as a result of the administration of ACTH. As in the mammals (Selye and Stone, 1950), ACTH injection leads to an increase in the number of eosinophils in the circulating blood, and it seems possible that the small lipid cells may differentiate into eosinophil leucocytes which are so essentially like them in structure and staining reactions. Selye and Stone remark that 'many observations suggest some intimate relationship between the adrenal cortex and haemopoiesis'. Furthermore, they state that in their experiments, which involved the injection of anterior pituitary extracts and sodium thyroxinate, haemopoietic tissue appears in the adrenal, and appears to be formed, at least in part, by the direct metaplasia of typical adrenal cortical cells into haemopoietic cells.

The phagocytes of the sinusoidal system are found in two principal forms, one the dendritic, whose chief function seems to be to phagocytize the extracellular phospholipid droplets. After active secretion by the lipid cells many of the phagocytes are found to be filled with the fine phospholipid droplets, and, on the other hand, after the 'blocking' of secretion from this tissue by hypophysectomy, they contain virtually no lipid.

The second type, the rounded form, takes up the pigment resulting from the breakdown of the large lipid cells, after their secretion of phospholipid. The ultimate destination of this type of phagocyte appears to be its incorporation into the round pigment cells, which are in fact syncytial structures. The evidence therefore from the lungfish is that these cells form not only a vascular system, but also take part in the transfer of material within the peri-renal organ.

In the absence of physiological information which could be correlated with

these microscopical observations, no scheme of the metabolism of the peri-renal tissue can be proposed. But the observations of the reaction of the cells to changes known to be part of the pituitary-adrenal system of higher animals suggest that further study of the lungfish may throw light on the organization of this system.

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A Note on Insect Wing Veins and their Tracheae

By JOHN SMART

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With one plate (fig. 3)

Summary

Experiments involving interference with the tracheae of the nymphal fore-wing pad of the American cockroach (*Periplaneta americana* (Linn.)) are described and the results discussed. It is concluded that the pattern of the tracheae is not immutable and so cannot be regarded as fundamental in determining the pattern of the imaginal wing-venation; the pattern may, however, continue to be used, in the first instance, as a guide to the more fundamental pattern of the lacunae that precede the veins.

Introduction

DURING the past half-century or so the Comstock-Needham system of nomenclature for insect wing veins has become generally accepted except where, in some cases, older special systems have been retained for convenience in works aiming mainly at the identification of species (e.g. Schiner's system in Diptera).

The theoretical foundations of the Comstock-Needham system (Comstock, 1918; Needham, 1935, 1952) are:

(1) A belief that the wings of all insects are homologous organs.

(2) A belief that the main longitudinal veins at least are homologous and that it should therefore be possible to discern a phylogenetic connexion amongst the venation patterns of all insect wings.

(3) A belief that the main longitudinal veins are, or at least were at one time in their phylogenetic history, laid down by the interaction of the wing epidermis on longitudinal tracheae.

(4) A belief that the general pattern of the venation is governed by the pattern of these tracheae and that this tracheal pattern, though subject to minor variation, is relatively constant in any given species and has certain consistencies in higher classificatory categories.

(5) A belief that when homologies of the venation cannot be determined by inspection of imaginal wings, they can be determined by reference to the pattern of the tracheal system in the nymphal wing-pad or the pupal wing.

Modifications of the Comstock-Needham system of vein nomenclature have been proposed at various times. Some, such as those of Tillyard (1919) and Snodgrass (1935), have met with general acceptance; others, such as those of Martynov (1925), Forbes (1932), and Zallesky (1944), have not.

In the past most work on the tracheal systems of nymphal or pupal wing-pads was done by examination of the wing-pad in glycerine or glycerine-jelly. Such preparations were impermanent. Permanent preparations of the tracheal system can now be made by the use of a technique devised by Wigglesworth

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(1950) for the injection of the tracheal system with a black mass of cobalt or lead sulphide (fig. 1).

Smart (1951) discussed the application of the Comstock-Needham system of nomenclature to the wing-venation of the American cockroach (*Periplaneta americana* (Linn.), using this injection technique for examination of the tracheae of the nymphal wing-pads. While this work was being done, some

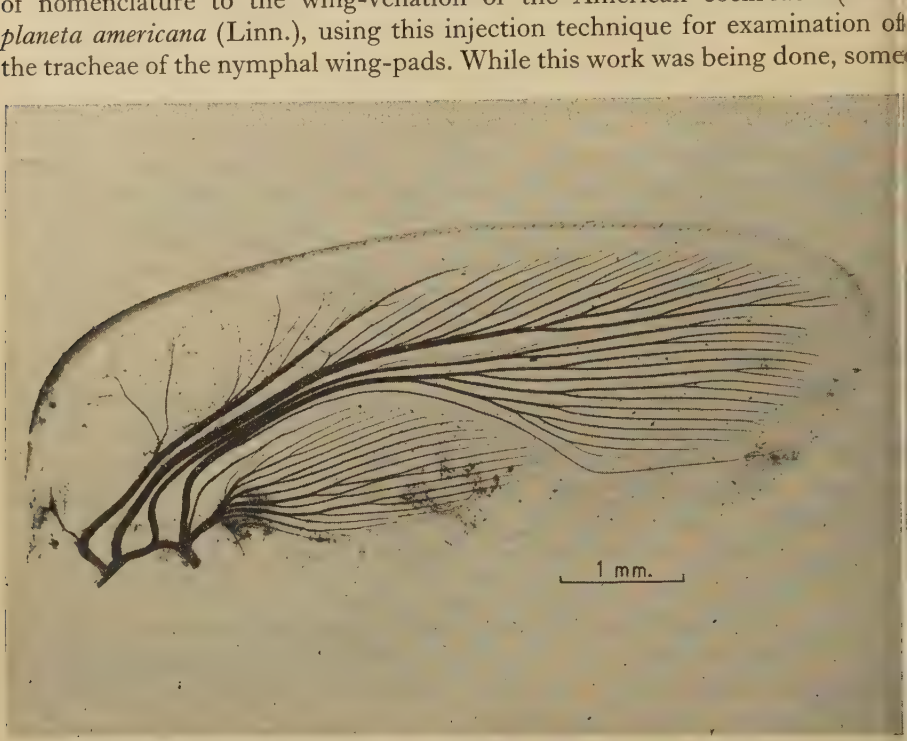

FIG. 1. Fore-wing pad of a normal last nymphal instar of *Periplaneta americana*, with the tracheae injected and viewed by transmitted light.

observations were made on the details of the tracheal system and the relationship of the tracheae to the veins.

The present note is concerned with observations on the results of operational interference with the tracheae in the fore-wing pad of nymphs of the American cockroach.

OBSERVATIONS

A fore-wing (tegmen) pad of a last-stage nymph of the American cockroach is shown in fig. 1; this specimen shows the normal pattern of the tracheation. Fig. 3 shows this tracheation with the Comstock-Needham nomenclature applied to the veins as in Smart (1951). Smart (1951) accepted Snodgrass's (1935) modifications of the Comstock-Needham nomenclature. Snodgrass (1935) proposed the name postcubitus (Pcu) for the first anal vein of the Comstock-Needham system and suggested that the subsequent anal veins of the Comstock-Needham system should be called vannal veins (V).

One of the most constant features of the tracheal pattern in normal nymphs

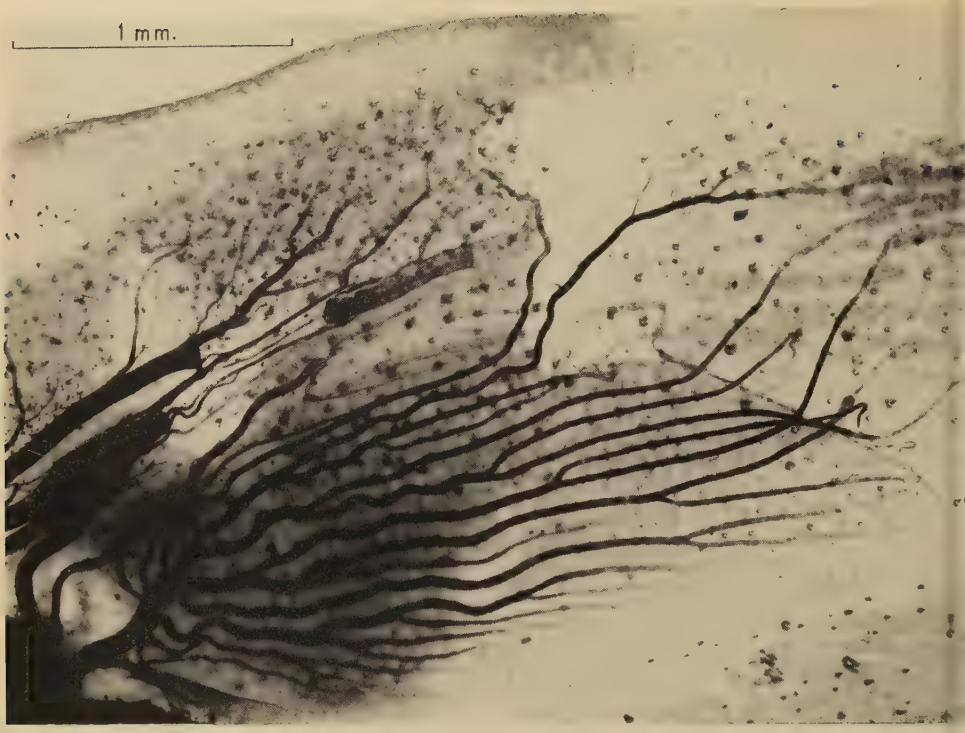

FIG. 3 A

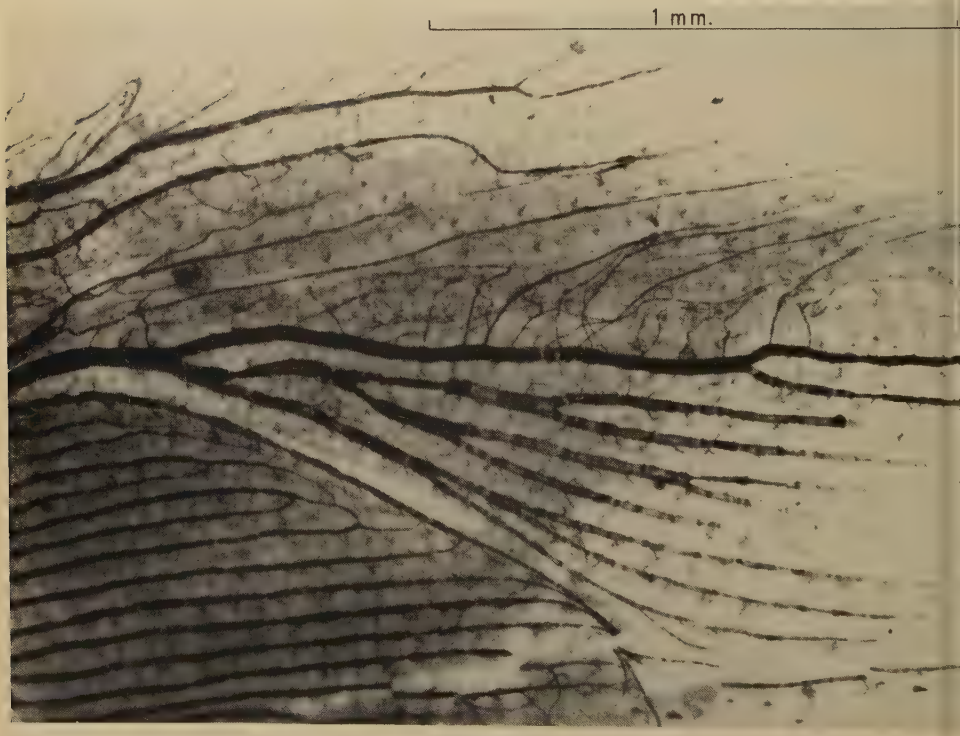

FIG. 3 B

J. SMART

is the way in which the area tracheated by Pcu and V is circumscribed by the trachea of Cu_2. This area is destined to be the clearly defined vannus of the imaginal tegmen.

The well-developed antero-pectinate branching of R may also be noted.

In the experimental case shown in fig. 3, A, a recently moulted penultimate-stage nymph of the American cockroach had the tracheae Sc, R, M, and Cu of the anterior (tegmen) wing-pad severed by the insertion through the cuticle of a fine scalpel, while the nymph was still in a teneral condition. The nymph was allowed to recover, feed, and grow. The wound healed and the nymph eventually moulted and entered the ultimate nymphal stage. It was

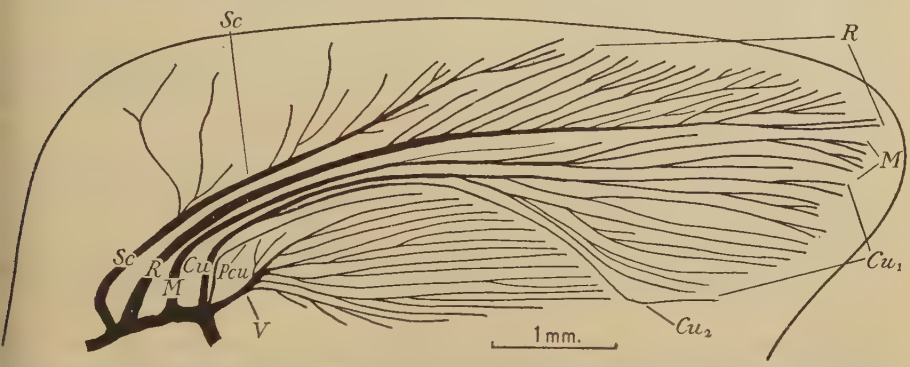

FIG. 2. Fore-wing pad of last nymphal instar of *Periplaneta americana*; the tracheae labelled with the Comstock-Needham system of venation nomenclature (as modified by Snodgrass). Conventional abbreviations for venation nomenclature using the Comstock-Needham system as modified by Snodgrass are: C = costa; Sc = subcosta; R = radius; M = media, Cu = cubitus; Pcu = postcubitus (= first anal of original Comstock-Needham notation); V = vannal (= second and subsequent anals of original Comstock-Needham notation).

then killed as soon as the cuticle had hardened, the tracheae injected, and the wing-pad mounted in balsam. A portion of this wing-pad is shown in fig. 3, A.

The following points should be noted:

The stumps of the severed Sc, R, M, and Cu tracheae have healed over and have a number of fine new tracheae growing out from them.

Some of these new tracheae from the stumps have grown out and joined themselves to the severed distal part of R and have retracheated it, allowing some of the injection mass to reach and pass into this severed part.

Trachea Pcu has increased in diameter and developed at least one small branch; it is normally a simple unbranched trachea.

Tracheae of the vannal area have grown right out beyond their normal area into the other areas that have been deprived of their normal tracheation by the experimental severance of the large anterior tracheae.

FIG. 3 (plate).

A and B both represent parts of the fore-wing pads of last nymphal instars of specimens of *Periplaneta americana*, showing the condition of the tracheae consequent on the experimental interference described in the text.

These outgrowing vannal tracheae have crossed the paths of Cu, M, and in one case R.

These outgrowing vannal tracheae are larger in diameter than in the normal wing-pad.

These outgrowing tracheae cross over each other without effecting a junction.

Branches develop on these outgrowing vannal tracheae; they are normally furnished with only very fine branches.

The ends of the outgrowing tracheae and their branches seem to turn into and then follow along the vein paths of the deprived areas. Note especially the second outgrowing vannal trachea which itself turns into the path of R and then sends out antero-pectinate branches.

The second case, shown in fig. 3, B, is of a similar experiment on a similar animal except that only M and R tracheae were severed. The severance of R was distal to the first main antero-pectinate branch thereof.

The following points should be noted:

A number of fine new tracheae have grown out from the stumps of M and R.

The new tracheae growing out from the stump of M have run up into the paths of the antero-pectinate branches of R that have been deprived of their tracheation.

Cu_1 has developed a great many small new anterior branch tracheae, the majority of which run up into the paths of the antero-pectinate branches of R but some of which turn into the path of M and/or the main path of R.

Discussion

The results noted above indicate that:

There is considerable plasticity latent in the tracheal system of the nymphal wing-pad.

The tracheal system responds to the detracheation of an area of the wing-pad by retracheating it by additional development of surviving tracheae.

Additional development consists, first, of an increase in length and diameter of certain surviving tracheae.

Secondly, the additional growth may take the form of extra branching of the tracheae quite different from their normal pattern.

After growing towards the detracheated area the developing tracheae and their branches tend to follow the paths up which the old tracheae passed (vein lacunae) rather than new paths of their own across the lacunae.

It is not necessarily the nearest tracheae that retracheate a detracheated area.

The developing tracheae will cross each other without, apparently, joining or connecting to each other on their way to the deprived detracheated area.

On the other hand, some of these developing tracheae join up with severed parts of the tracheal system in a manner indicative of effective respiratory reconnexion.

In connexion with these static observations on the developmental responses of the tracheae it may be noted that Wigglesworth (1954) has discussed the details of the growth and regeneration of the tracheal system not only in tissues deprived of their original tracheation but also in tissues subjected to a subnormal oxygen tension. He includes observations on the wing-pad of the bug *Rhodnius*: subnormal oxygen tension resulting in development of additional tracheation.

Conclusions

The pattern of the tracheal system in the wing is not immutable.

The pattern of the tracheae in the nymphal wing-pad or the pupal wing cannot be taken as fundamental in determining the homologies of the wing-veins. It may, however, be an excellent guide thereto in so far as the main tracheae normally pass along the lacunae that precede the veins in the wing-pad or pupal wing.

The condition of one trachea crossing another, a feature of the tracheation of the wings of the anisopterid dragonflies, can be induced experimentally.

The situation produced, by the described experimental interference, in the nymphal wing-pads reminds one of the concept of the developmental or morphogenetic fields utilized by vertebrate embryologists.

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Observations on the Stomach and Digestive Diverticula of the Lamellibranchia

II. The Nuculidae

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Summary

The anatomy and histology of the stomach and digestive diverticula and the physiology of digestion in the Nuculidae have been investigated.

The stomach is relatively large and possesses a well-developed style sac, although a rod-like crystalline style is absent. Sorting areas are present in the dorsal region of the stomach but the ciliary currents are relatively weak and have little effect on the ingested material.

The digestive diverticula consist of three regions, the physiological relations of which have not been fully elucidated. The three regions are: (*a*) a much-branched system of non-ciliated main ducts, the epithelium of which shows a well-developed brush-border; (*b*) short, unbranched ciliated secondary ducts; (*c*) irregularly branched blindly ending tubules. The epithelium of the tubules is divided into two regions, one composed of large cells containing numerous conspicuous granules and the other of less frequent and smaller darkly staining cells. Each darkly staining cell bears a single flagellum.

Separate mucous glands cannot be demonstrated in any part of the stomach or digestive diverticula.

The epithelium of the style sac secretes an amylase and a lipase. Extracts of the digestive diverticula contain an amylase, a lipase, and a protease.

Unlike the majority of lamellibranchs, fluid and solid particles do *not* appear to enter the digestive diverticula from the stomach and in no part of the gut or of the diverticula is there ever any evidence of intracellular digestion. It is suggested that in the Nuculidae digestion is exclusively extra-cellular and confined to the stomach. The soluble products are absorbed by the epithelium of the stomach and first part of the intestine.

The diverticula of the rest of the Lamellibranchia are compared with those of the Nuculidae. While the Nuculidae are in many respects primitive lamellibranchs, the conclusion is reached that the digestive diverticula present many specialized features possibly correlated with the method of feeding.

Introduction

IT has been suggested by many writers (Stempell, 1898; Heath, 1937; Yonge, 1939) that the protobranchiate molluscs are in many respects the most primitive living lamellibranchs and the structure and physiology of the gut in these animals is therefore of considerable interest. A study of the digestive diverticula of the Anisomyaria and Eulamellibranchia revealed certain features hitherto undescribed (Owen, 1955), and the reason for this present study was to determine whether similar features are present in the Taxodonta. However, variations in the structure and function of these organs both within the Taxodonta and when compared with the Anisomyaria and Eulamellibranchia are considerable, and as a consequence of this it was

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desirable to restrict the initial study to a single family. A number of species belonging to the Nuculidae—including the largest British species, *Nucula sulcata*—occur in the Clyde sea area (Allen, 1954), and since Yonge (1939) concluded that the members of this family are the least specialized of the protobranch molluscs, the choice of family was easily made. While all four species which occur in the Clyde sea area, *N. sulcata*, *M. turgida*, *N. tenuis* and *N. nucleus*, were examined, the structure and function of the stomach and digestive diverticula are described with particular reference to *N. sulcata* and the minor differences which occur in the other species noted where necessary.

Dissections of the stomach and digestive diverticula were carried out on living specimens and the course of the ciliary currents followed by means of fine carborundum power and colloidal graphite ('Aquadag'). Bouin's fluid and a modified Bouin-Duboscq's fluid (Atkins, 1937) were satisfactory fixatives for general histological purposes. Sections 2–8 μ thick were stained either in Heidenhain's 'Azan' or in Heidenhain's iron haematoxylin, alcian blue 8GN (replaces alcian blue 8GS, Steedman, 1950), and orange G in clove oil; Mayer's haemalum was frequently used in place of Heidenhain's iron haematoxylin. To prevent the growth of mould, the alcian blue contained 0·5% propylene phenoxetol (Owen and Steedman, 1956). Before embedding in ester wax the material was cleared in monochlorisothymol (Steedman unpublished work).

The Morphology of the Stomach

In describing the stomach of *N. sulcata* the nomenclature used by Graham (1949), Owen (1953), and Purchon (1954, 1955) has been followed where possible. The external appearance of the stomach and style sac when viewed from the right and left sides respectively is shown in fig. 1, A and B. As in the majority of the Nuculidae (Yonge, 1939), the structure is divided into a globular dorsal region and an elongated, reddish region extending ventrally into the foot; the dorsal region is further divided into dark green and cream coloured portions. As both Yonge (1939) and Graham (1949) have already emphasized, the tapering ventral region is homologous with the style sac of other Lamellibranchia, while the dorsal region is the stomach proper. The oesophagus enters the stomach antero-dorsally and a little to the right of the mid-line, while the intestine emerges from the style sac ventrally and passes dorsally posterior to the stomach. As in the Anisomyaria and Eulamellibranchia a well-developed dorsal hood ('dorsal pouch' of Graham, 1949) extends on to the left wall of the stomach to end blindly on the left dorsal side near the aperture of the left duct. Three ducts enter the stomach from the diverticula, one on the left and two side by side near the mid-line ventral to the oesophagus. To the left of the oesophageal aperture a small pouch of caecum extends dorsally to end blindly to the right of the mid-dorsal line. A similar pouch on the right side of the oesophageal aperture is formed by the anterior region of the posterior sorting area which is seen through the wall of the stomach and extends over most of the right side.

The drawing of the internal features of the stomach and style sac (fig. 2) is he result of observations on a number of specimens, since no matter how carefully the stomach is opened some part of the internal anatomy is invariably ost or distorted. Extending ventrally from the stomach is the combined style ac and intestinal groove. Along the right anterior wall of the style sac run wo ciliated ridges, designated by Graham (1949) in his description of the

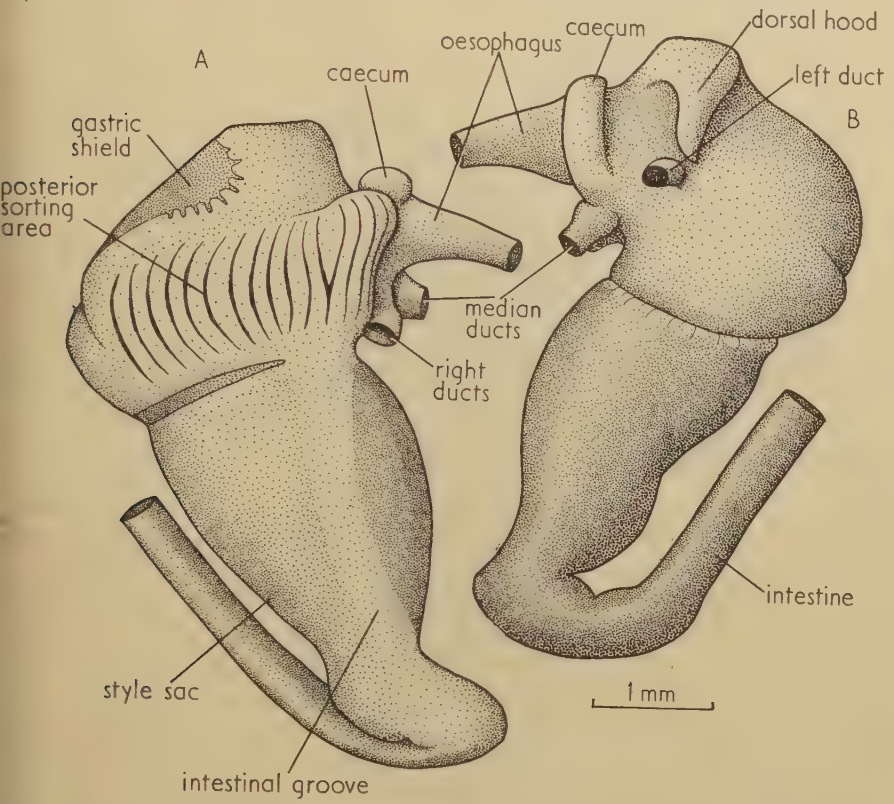


FIG. 1. The external appearance of the stomach of *N. sulcata* viewed from A, the right side; B, the left side.

stomach of *N. hanleyi* as the major and minor typhlosoles, with the intestinal groove between. Ventrally, at the junction of the style sac with the mid-gut, the two typhlosoles give rise to numerous longitudinal ridges which run the length of this portion of the intestine; the intestinal groove is continuous with the grooves between these ridges. On reaching the stomach dorsally the two typhlosoles separate, the major crossing on to the left wall of the stomach to end at the aperture of the left duct, while the minor typhlosole runs posteriorly along the ventral region of the right wall before curving dorsally and anteriorly to end near the oesophageal aperture. It thus forms a U-shaped structure on the right wall of the stomach, the open part of the U being directed anteriorly

and partially closed by the well-developed fold surrounding the oesophageal aperture. As in *N. hanleyi* (Graham, 1949), the minor typhlosole encloses a regular series of folds and ridges which run dorso-ventrally and extend over almost the entire right-hand wall of the stomach. In *N. sulcata*, a somewhat narrower but similar series of grooves and ridges, the rejection tract, runs

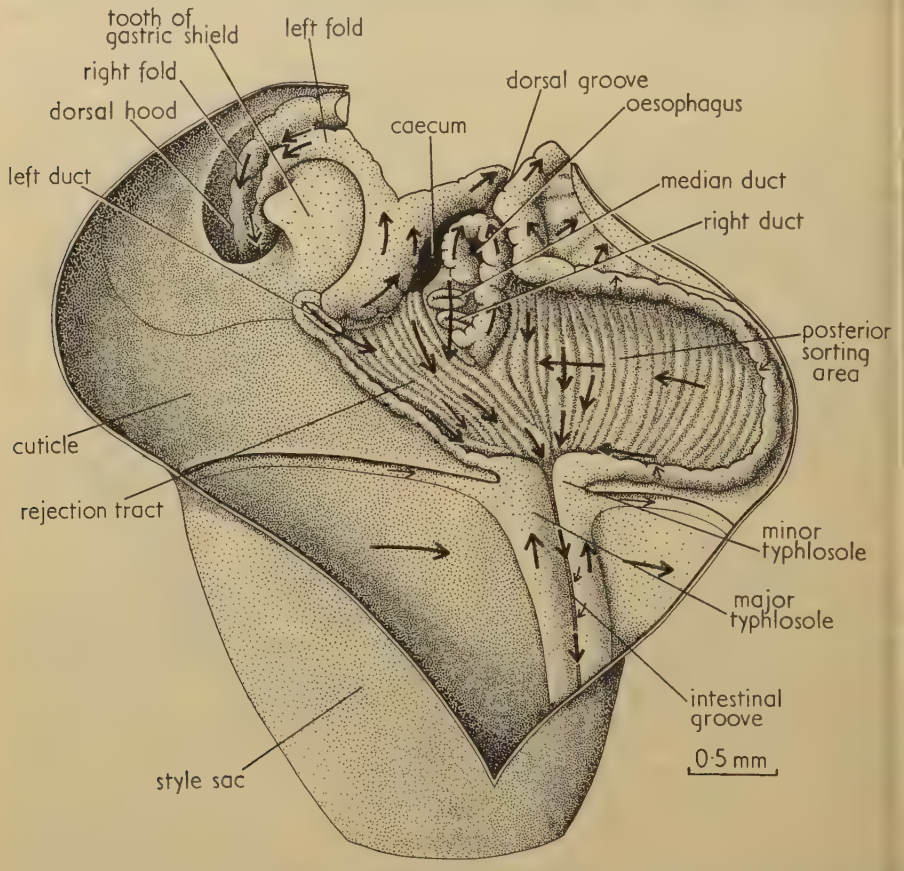


FIG. 2. The stomach of *N. sulcata* opened by a dorsal and posterior incision. The arrows indicate the direction of the ciliary currents.

across the anterior floor of the stomach and extends on to the anterior left wall to enter the slit-like aperture of the caecum to the left of the oesophageal opening. In *N. turgida* the rejection tract consists of a single groove, more or less continuous with the intestinal groove of the style sac and extending to the mouth of the caecum, while in *N. tenuis* and *N. nucleus* conditions are similar to those figured for *N. hanleyi* by Graham (1949). In all four species a pouch-like caecum, not shown by Graham in his drawing of the stomach of *N. hanleyi* is present to the left of the oesophageal aperture; the wall of this caecum is produced into a series of folds and ridges.

Two well-developed folds, with a groove between, enter the dorsal hood

The left fold arises on the left wall of the stomach near the aperture of the left duct and runs dorsally, forming the posterior margin of the opening of the caecum, before extending posteriorly to enter the dorsal hood. The right-hand fold is more or less continuous with the minor typhlosole and arises to the right of the oesophageal aperture; it extends posteriorly over the roof of the stomach before curving to the left to enter the dorsal hood. As a result of this disposition of the two folds, the groove between arises from a position dorsal to the oesophageal aperture. Both the folds and the groove run the length of the dorsal hood to terminate at the blind-ending tip.

The remainder of the gastric wall, consisting of the greater part of the left side, the posterior region of the dorsal hood, and a narrow tongue-like projection extending on to the right wall at the junction of the style sac with the stomach, is dark-coloured and non-ciliated, being covered by a layer of cuticle secreted by the underlying epithelium. The cuticle which surrounds the opening of the dorsal hood and extends over the roof of the stomach on to the right dorsal wall is tougher than that covering the remainder of the gastric wall, and can be removed intact. As suggested by Yonge (1939), this region is homologous with the gastric shield of other Lamellibranchia. Anterior to the opening of the dorsal hood the cuticle of the shield is raised to form a prominent tooth-like structure.

The epithelium of the stomach can be divided into three distinct regions: (*a*) the style sac, (*b*) the major and minor typhlosoles together with the ciliated regions of the stomach, and (*c*) the epithelium underlying the cuticle. The wall of the style sac consists of a smooth epithelium, some 50 to 60 μ deep, bearing dense bristle-like cilia, and the distal region of the cells contains numerous small pigment granules. As described by Yonge (1939) this epithelium corresponds in all respects with the style sac epithelium of other lamellibranchs. The remaining ciliated epithelium is composed of typical columnar ciliated cells of varying heights which produce the grooves and ridges of the stomach wall. Small pigment granules are again present in the distal region of the cells. Although the stomach contents are invariably compacted in a mucus-like substance, no mucous glands are to be found either in the epithelium or lying in the tissue below. Separate mucous glands cannot be demonstrated in any part of the gut.

The epithelium underlying the cuticle is formed of tall slender columnar cells ranging from 100 μ in height to as much as 400 μ under the tooth-like structure of the gastric shield; the height of the tooth above the surface of the gastric shield is almost exclusively due to this increase in height of the underlying epithelium. The cells possess elongated nuclei in the basal third of the cytoplasm and the distal region is frequently packed with spherical pigment granules up to 4 μ in diameter, yellowish green in fresh material and colouring with Sudan black B in wax-embedded sections. They are responsible for the dark green colour of the epithelium underlying the cuticle and as suggested by Yonge (1939) are probably excretory.

Underlying all three types of epithelium is a layer of collagen through which

runs a system of fine anastomosing fibres, 0·5 μ or less in diameter. In fresh preparations these fibres are brown and they retain this colour even after fixation and subsequent wax embedding. As a consequence of this and of their small size they appear black in section and it is difficult to determine their staining properties, but it seems likely that they are reticular fibres serving a skeletal function. External to the layer of collagen is a system of smooth muscle-fibres. Circular fibres predominate at the junction of the style sac with the intestine and form a sphincter muscle. Both stomach and style sac are invested in a loose mesh-work of connective tissue which is frequently packed with small rounded granules 1–2 μ in diameter, staining black with Heidenhain's haematoxylin and red with Azan. They stain intensely with mercuric bromphenol blue (Mazia, Brewer, and Alfert, 1953) and are evidently protein in nature. Similar granules have been described in chitons (Fretter, 1937) and in the gastropod, *Carychium* (Morton, 1955).

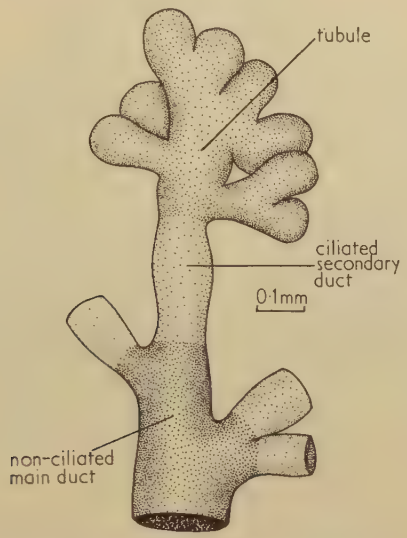
The pattern of ciliary activity within the style sac and stomach is indicated by arrows in fig. 2. The dense bristle-like cilia of the style sac beat in the same direction as in other Lamellibranchia, i.e. a clockwise direction when viewed from above. Along the intestinal groove the ciliary currents are directed ventrally towards the mid-gut while on the major and minor typhlosoles weak ciliary currents are directed dorsally towards the stomach; at the sides of the intestinal groove the cilia beat into the groove.

Over the ridged and grooved area of the right wall of the stomach the ciliary currents are similar to those of the posterior sorting area of other Lamellibranchia. The cilia in the groove beat ventrally and drain into the intestinal groove while those on the crests of the ridges beat anteriorly above and across the grooves. The currents produced by the cilia on the crests are noticeably weaker than those produced by the cilia in the grooves. The ciliary currents over the prominent fold which surrounds the oesophageal aperture are directed dorsally and drain into the dorsal groove. In the groove, and also over both the right and left folds, the ciliary currents are directed towards the dorsal hood in *N. sulcata*, *N. turgida*, and *N. tenuis*. Thus, unlike *N. hanleyi* (Graham, 1949), there are *no* ciliary currents beating out of the dorsal hood. Although the grooves and ridges which extend over the anterior floor of the stomach and on to the left wall appear to be an extension of the sorting area of the right wall, the ciliary currents of this region do not exercise any selection of the stomach contents. The cilia both in the grooves and on the ridges are all directed towards the intestinal groove of the style sac. This region is probably homologous with the extension of the intestinal groove across the floor of the stomach in other Lamellibranchia, but to avoid confusion with the intestinal groove of the style sac the term 'rejection tract' is used. A second sorting area is present within the small caecum to the left of the oesophageal aperture, the wall of which also bears a series of folds and grooves. The ciliary currents in the grooves are directed ventrally towards the rejection tract while the cilia on the crests of the folds beat across the grooves and towards the dorsal groove and folds. Again the ciliary currents on the ridges are noticeably weaker than

hose in the grooves. Finally, the slit-like entrance to each of the three ducts eading to the digestive diverticula is surrounded by long, fine cilia which beat iolently away from the duct and towards the rejection tract.

The Digestive Diverticula

As in the majority of lamellibranchs, the digestive diverticula surround the greater part of the stomach and consist of numerous blindly ending tubules which communicate with the stomach by a well-developed system of ducts. In all the species examined, the tubules and ducts are packed loosely together with little connective tissue between them, and the various regions of the diverticula can be readily recognized in dissections of fresh animals (fig. 3).



IG. 3. A portion of the digestive diverticula of *N. sulcata* showing the darkly coloured main ducts, the lightly coloured secondary ducts, and the brown, blindly ending tubules.

While the main ducts which communicate with the stomach are a dark olive reen and branch repeatedly, the short secondary ducts to which they give se are light yellow or cream-colour. They terminate in blindly ending ubules which are brownish and have the form of irregularly branched elongate ubes with numerous saccular outgrowths. The layer of collagen and the ystem of reticular fibres underlying the epithelium of the stomach is con-nued beneath the epithelia of the main and secondary ducts and the tubules, ut they are progressively less well developed as they approach the blindly nding tubules. Occasional fine muscle fibres are also present in all three egions, but they are never as well developed as in the corresponding regions f the diverticula of the Anisomyaria and Eulamellibranchia (Owen, 1955).

he main ducts

Both Heath (1937) and Yonge (1939) describe the Nuculidae as possessing nly two ducts from the diverticula into the stomach, but Graham (1949)

found that in all the specimens of *N. hanleyi* examined by him there wer three ducts, two on the right and one on the left. In *N. sulcata*, *N. tenuis*, *N turgida*, and *N. nucleus*, three ducts enter the stomach. One opens on the le wall ventral to the gastric shield and serves the left mass of the diverticul while the remaining two enter the stomach side by side ventral to the oes phageal opening and slightly to the left of the mid-line (figs. 1 and 2). C these two ducts, the left one serves the median anterior mass of the diverticul while the right duct, which bends posteriorly immediately on leaving th stomach, serves the right mass.

The ciliated epithelium of the stomach projects only a short distance int

FIG. 4. *N. sulcata*, the epithelium of the main ducts of the digestive diverticula during t 'extrusion' phase.

the main ducts and is replaced by a non-ciliated epithelium ranging from 2 to 40 μ in height, present throughout the main ducts (fig. 4). It consists only one type of cell with spherical or slightly oval nuclei and at the fr margin a well-developed brush-border which extends 8–10 μ into the lume of the duct. In fresh preparations of the diverticula squeezed out under coverslip this epithelium is easily disrupted, and so prominent is the brus border that each cell has the appearance of a miniature shaving brush. dissections of fresh material, the internal surface of the main ducts possesses bright bluish-green iridescence probably due to the reflexion of light fro the surface of the brush-border. In the region above the nuclei occur numero pigment granules, their size varying from 5 μ to less than 0·5 μ. In fre material the granules are yellowish green and give the olive green appearan which the main ducts present in the living animal and which contrasts wi

he much lighter shade of the secondary ducts where the pigment granules re smaller and fewer. The granules are some form of lipo-pigment and colour trongly with Sudan black B even after impregnation with ester wax. They re invariably reduced in size after Bouin or alcoholic fixation, while after Lewitsky's fluid (Flemming without acetic) or Helly's fluid their appearance s very similar to that in life. While it is difficult to say what part they play in he activities of the cell it is probable that they are excretory since many of he lipo-pigments are formed as by-products of lipid metabolism. Certainly n frozen sections the basal region of the cells is full of lipid material which annot be demonstrated in wax sections.

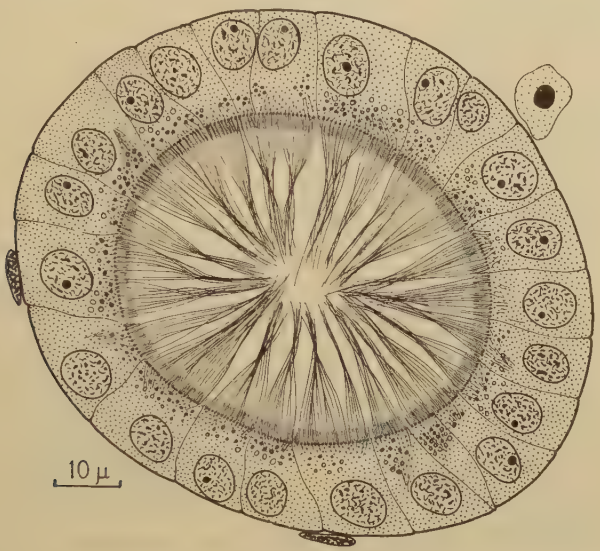


Fig. 5. A transverse section through a secondary duct of the digestive diverticula of *N. sulcata*.

The cells appear to undergo a secretory or excretory cycle, since the appearance of the epithelium varies considerably although more or less constant in any one animal. Fig. 4 illustrates the epithelium during the 'extrusion' phase. Numerous vesicular outpushings, into which the pigment granules are extruded, are being nipped off from the surface of the epithelium. In wax-embedded sections the lumen of the ducts frequently contains numerous vesicles either apparently empty or containing relatively small amounts of granular material that stains with iron haematoxylin. Presumably the staining properties of the granules are quickly lost after extrusion from the epithelium. Alternating with these extrusion phases the free margin of the epithelium is smooth and vesicular outpushings are absent.

The secondary duct

In contrast to the main ducts the epithelium of the secondary ducts is uniformly covered with a dense coat of long cilia, some 20 to 25 μ in length, which extend almost to the middle of the lumen (fig. 5). They beat towards the

main ducts with a marked clockwise trend when viewed from the tubules. *There are no cilia beating towards the tubules.* Again only one kind of cell is present, ranging from 15 to 25 μ in height and containing large, slightly oval nuclei, each with a prominent nucleolus, lying in the mid-region of the cytoplasm. Pigment granules are present in the region above the nucleus, but they are smaller and fewer than those in the epithelium of the main ducts.

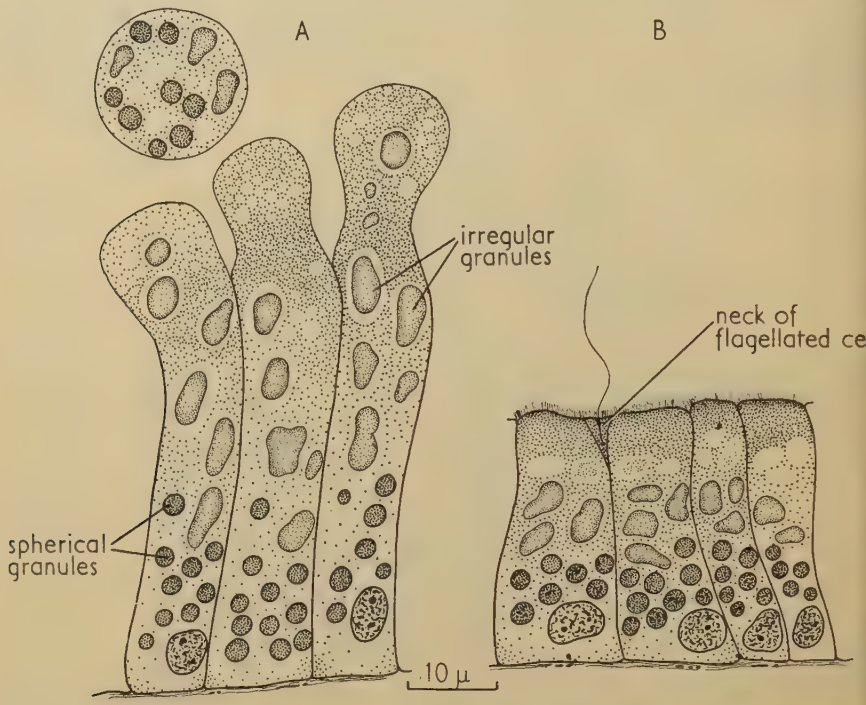


FIG. 6. Different phases of the granule cells lining the tubules of the digestive diverticula of *N. sulcata.*

The tubules

The blindly ending tubules of the diverticula are round or oval in cross section and two types of cell may be distinguished. Most numerous are those ranging from 30 to 80 μ in height, while the free margin of the cells varies from regular to pseudopodial in outline (fig. 6, A and B). The nuclei are rounded and somewhat ovoid, basal in position, and each with a small nucleolus. Numerous brown, rounded granules, occasionally showing internal granulation, fill most of the cell. Those in the basal region are fairly regular in outline. They range from 2 to 4 μ in diameter and stain light blue with Mallory's. In the middle region the granules are larger, 5–7 μ in diameter, fewer in number and irregular in outline; they are coloured dark blue or yellow after staining with Mallory's. Staining with haemalum, alcian blue, and orange G in clove oil indicates that the smaller and more numerous basal granules are surrounded by a 'mucous' membrane staining with alcian blue while the contents are

stained with haemalum. The larger irregular granules are coloured yellow. In sections treated with PAS (Hotchkiss) the larger granules give a strongly positive reaction which decreases in intensity in the basal granules; control sections treated with diastase gave similar results. The greatest differentiation between the two types of granules is obtained after treatment with Ritter and Oleson's (1950) modification of Hale's (1946) method for acid mucopolysaccharides. The smaller spherical basal granules are coloured blue while the larger irregular granules in the middle region of the cell are red (for convenience the two types of granules will be described as 'spherical' and 'irregular' respectively). Pearse (1953) has, however, criticized the low specificity of Hale's method and the striking results obtained are of little value in determining the nature of these granules. It would appear that the smaller and more numerous spherical granules are surrounded by an acid mucous membrane which is absent from the larger, irregular granules. Beneath the free margin of the cell is a region of dense cytoplasm followed by a small number of vacuoles which either appear empty or occasionally contain an irregular mass of faintly staining material.

The second type of cell found in the tubules is much less common than those already described (fig. 7, A and B). They are found scattered irregularly in small clusters of up to a dozen cells round the lumen of the tubules and are readily distinguished from the larger 'granule' cells by their larger nuclei and more darkly staining cytoplasm. They are narrow at the distal end but broaden out markedly at the base so as to be triangular in outline. The nucleus is basal in position, circular in outline, and in contrast to the nuclei of the granule cells contains a very prominent nucleolus. The greater part of the cell is filled with dense, darkly staining cytoplasm frequently showing traces of intracellular fibres and including a varying number of irregular granular masses.

In the Eulamellibranchia, flagella are readily observed in fresh preparations of the diverticula moving with a languid, undulating beat within the tubules. On only a few occasions were similar flagella observed in the Nuculidae, yet they are undoubtedly present in all specimens. In the eulamellibranchs, the darkly staining cells of the tubules are flagellated (Owen, 1955), and this is also true of the Nuculidae, but unlike the Eulamellibranchia, each darkly staining cell bears only a single flagellum (fig. 7). As a consequence of this, basal granules and flagella are difficult to observe in section. After fixation with Bouin-Duboscq's fluid and staining with Heidenhain's iron haematoxylin, a single flagellum and basal granule can frequently be seen at the distal ends of the darkly staining cells. Because of the narrow neck-like shape of the upper portion of these cells it was at first difficult to be certain that this appearance was not due to the membranes of the underlying cells. Fortunately, one section passed transversely through the distal region of a clump of darkly staining cells and showed clearly the boundary of each cell with a single basal granule or flagellar shaft in the centre. In previous accounts of the diverticula of Lamellibranchia, the vibratile structures occurring within the tubules have been described as cilia, but unlike the cilia occurring elsewhere in the lamellibranch

body they beat with an undulating or sinusoidal movement rather than a simple flexing or bending motion; and they do not exhibit metachronal rhythm (Owen, 1955). These observations on their mode of beating and distribution (i.e. only one or, at most, a few to a cell) indicate that they may be more correctly termed flagella.

The appearance of both the granule cells and flagellated cells, although more or less constant in any one animal, varies a good deal, suggesting that a phase change occurs. Two extremes of the phase changes of the granule cells are shown in fig. 6, A and B. In fig. 6, B the free margin of the cells is flat and

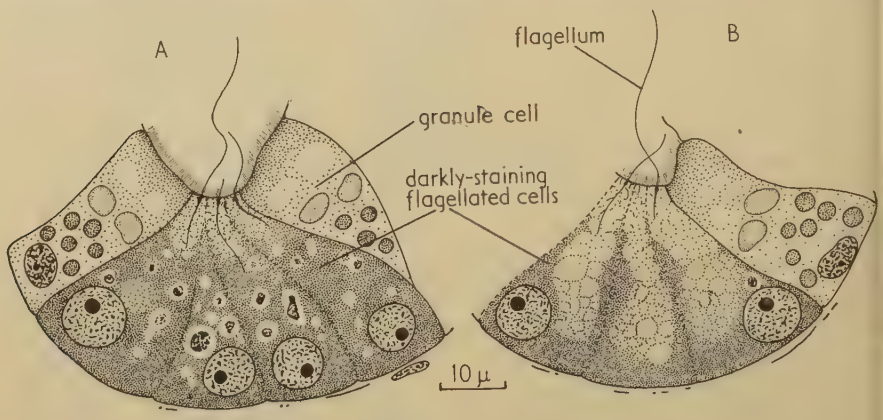


FIG. 7. Different phases of the darkly staining, flagellated cells of the tubules of *N. sulcata*.

regular in outline and presents a fibrillar appearance similar to that of a poorly developed brush-border. Beneath the free margin is a region of dense cytoplasm followed by one or two large vacuoles either containing an irregular mass of faintly staining material or apparently empty. In fig. 6, A the cells are taller and the distal ends rounded or club-shaped, the free margin no longer presenting a striated appearance. Large spheres some 15 to 20 μ in diameter, which contain numerous granular inclusions similar to those present within the cell, are freely constricted into the lumen. The various phases of the darkly staining cells show fewer differences than do those of the granule cells. As shown in fig. 7, A and B, the different phases suggest that material is built up within the cell and periodically discharged into the lumen of the tubule, aided possibly by the beating of the flagellum. Despite the general similarity in structure of both the granule cells and darkly staining cells to the cells which are found in the tubules of other lamellibranchs and gastropods, it has not been possible to determine their function with any degree of certainty. A discussion of their possible functions will be left until after the results of the various feeding experiments have been described.

Reserve Food Material

Apart from the protein spheres already described, reserve food is stored in the Nuculidae in the form of fat and glycogen. Considerable quantities of fa

ccur in the digestive diverticula. Fresh preparations of these organs pressed ut under a coverslip show numerous fat droplets. Although fat is present in ıost of the tissues, gelatine-embedded frozen sections coloured with Sudan V or Sudan black B show that it is most abundant in the lower third of the ells lining the main ducts and in the basal region of the granule cells of the ubules. In freshly caught animals, fat is abundant throughout the ciliated pithelium of the stomach and the first part of the intestine, but after 9 days' tarvation only traces remain at the base of the cells. This short period of tarvation has little effect on the amount of fat present in the diverticula. Glycogen is present in considerable quantity in all the tissues of the stomach nd digestive diverticula.

Feeding Experiments

The absorption of ingested material was tested by feeding the animals on uspensions in sea-water of iron saccharate, colloidal graphite (Aquadag), itanium dioxide, rice starch, and an emulsion of olive oil stained with waxoine red OS. (Feeding with glycogen was unsuccessful since the relatively hort period of starvation which was possible had little effect on the amount of glycogen occurring naturally in the tissues.) Animals were placed in these uspensions and fixed after definite periods; those fed on iron, in 10% formalin buffered to pH 7–7·4 (Lison, 1953), or in a mixture of Bouin's fluid and ammonium sulphide (Yonge, 1926). Iron was demonstrated by treating the ections for 20 minutes with the following mixture: 25 ml of 5% potassium erricyanide, 25 ml of 5% potassium ferrocyanide, and 50 ml of 2% hydrochloric acid. Animals fed with colloidal graphite and titanium dioxide were ixed in Bouin's fluid and stained with haemalum and orange G in clove oil. Starch-fed animals were also fixed in Bouin's fluid and the sections stained with PAS (Hotchkiss) and with Lugol's iodine. Animals fed with olive oil were fixed in Baker's formaldehyde-calcium, and gelatine-embedded frozen ections were employed.

It was at first assumed that digestion would be largely intracellular (Yonge, 1939), and the initial experiments, using colloidal graphite (particle size less than 0·5 μ), titanium dioxide (0·5–2 μ), and rice starch (4 μ), were designed to determine where and how intracellular digestion takes place. All three substances are readily ingested, so that the lumen of the stomach and of the intestine is frequently filled with the material employed. *Nevertheless no part of the gut or of the diverticula ever showed any evidence of intracellular digestion.* Furthermore, solid particles of the material present in the stomach were never present in the lumina of the ducts or of the tubules. Occasionally some solid material was present just within the entrance to the main ducts, but this may well have resulted from spasmodic muscular contractions during fixation. Caspers (1940) showed that specimens of *Nucula* freely suspended in water could ingest fine particles present in the water and he concluded that *Nucula* is a suspension feeder obtaining its food by means of the respiratory currents. As shown by the present series of feeding experiments, *Nucula* is certainly able

to ingest particles present in suspension in the water but, as demonstrated b Hirasàka (1927) and Yonge (1939), the Nuculidae normally lie beneath the sur face of the substrate and use the extensile palp proboscides to collect bottor deposits. Caspers's experiments are therefore of little value in determining th method of feeding of *Nucula* under natural conditions. As suggested b Jørgensen (1955), the gills of the Nuculidae are so small and the water current so weak that it is unlikely that suspended material plays a major role in nutri tion. The stomach contents of freshly caught animals also support the viev that the Nuculidae are essentially deposit feeders, since the bulk of the ingeste material consists of relatively large particles incapable of remaining in suspen sion in the water. Further feeding experiments were therefore carried out, th animals being placed on an adequate quantity of bottom material mixed wit small amounts of titanium dioxide and of rice starch. The animals soo buried themselves in the manner described by Yonge (1939), and subsequen examination of the gut contents revealed that they ingested considerabl quantities of material, including the titanium dioxide and rice starch. Agai there was no evidence of intracellular digestion and solid particles were neve present in the digestive diverticula.

As a consequence of these results, particular attention was paid to feedin experiments with iron saccharate and olive oil coloured with waxoline red OS Feeding experiments with iron saccharate appeared at first to confirm th results described by Yonge (1926), but examination of control animals fixe as soon as possible after being caught showed that the various tissues of th digestive system contain considerable amounts of inorganic iron. Further more, the bottom deposits ingested by the animal are also rich in inorgani iron, the contents of the lumen of the gut giving a definite blue reaction i sections tested for iron. The main site of iron in the stomach is the pigmen granules in the cells underlying the cuticle, while in a few specimens occasiona fine granules of iron are also present in the distal regions of the ciliated cells In the digestive diverticula a faint positive reaction for iron is frequently give by the pigment granules in the epithelium of the main and secondary ducts and by the inclusions of the darkly staining cells of the tubules. It is, however the spherical granules in the basal region of the granule cells of the tubule that invariably give the strongest reaction to tests for inorganic iron, th irregular granules being invariably negative.

After relatively short periods of starvation, the pigment granules of both th stomach and digestive diverticula show no trace of iron, while the reaction o the spherical granules in the tubule cells is less marked and frequentl negative. Thus before feeding with iron saccharate the animals were kept i filtered sea-water for 48 hours. Iron is ingested in great quantity, filling th stomach and appearing in the first part of the intestine in 6–8 hours, so tha the lumen appears as a solid sheet of blue in section. Iron is absorbed by th ciliated epithelium of the stomach and the first part of the intestine, the cell showing a diffuse blue colour. After 24 hours the spherical granules of th tubule cells present a uniform blue colour, but there is never any evidence o

a general absorption of granules of iron throughout the cytoplasm, nor any diffuse blueness in it. After 48 hours the pigment granules of the darkly staining cells of the tubules, of the ciliated cells of the secondary ducts, of the brush-border cells of the main ducts, and of both the ciliated epithelium and the epithelium underlying the cuticle of the stomach, all give a positive reaction for iron. The most striking difference from the normal condition is that shown by the darkly staining cells of the tubules, the cell inclusions giving an intense blue colour.

Gelatine-embedded frozen sections of animals fed with olive oil coloured with waxoline red OS show that red droplets of olive oil are readily ingested and the ciliated epithelium of the stomach and of the first part of the intestine appears a diffuse faint pink. There is never any evidence of coloured oil in the lumen and cells of the ducts and tubules of the diverticula.

From these observations it appears that digestion is entirely *extracellular* and that the soluble products of digestion are absorbed at the site of digestion. The role of the digestive diverticula is extremely difficult to evaluate. None of the feeding experiments provided satisfactory evidence of their being organs of absorption as in the Anisomyaria and Eulamellibranchia. Animals kept in filtered sea-water for 48 hours and injected with 0·1 ml of dilute soluble iron saccharate showed a distribution of iron similar to that shown after feeding with the substance. There was no diffuse blue colour in the ciliated epithelium of the stomach but the spherical granules of the large cells of the tubules and the pigment granules described above all gave a positive reaction to tests for iron. From the available evidence the digestive diverticula of the Nuculidae can only be regarded as organs of secretion and excretion. It is remarkable that while in all other lamellibranchs the digestive diverticula function as organs of absorption, they do not do so in the Nuculidae. Even allowing for the fact that the substances used in the feeding experiments are not in any way related to the natural food of the animals, and that the techniques employed may have failed to demonstrate low concentrations of soluble iron, it is difficult to appreciate how fluid could enter and circulate within the digestive diverticula of the Nuculidae. Cilia surrounding the openings of the main ducts into the stomach beat actively into the stomach, while within the diverticula, cilia are restricted to the short secondary ducts and beat *away* from the tubules. In such finely divided diverticula possessing an extensive duct system, it is unlikely that muscular activity could effect an efficient interchange of material between the stomach and the tubules, and it would almost certainly result in some solid particles entering the diverticula. This was never observed.

Enzymes

The only recorded investigation of the digestive enzymes of the gut of the Nuculidae is that given by Yonge (1939) for *N. turgida*. The stomach contents were tested for amylolytic activity with negative results. In this present work, and at the suggestion of Professor C. M. Yonge, the nature of the enzymes

of the digestive diverticula and of the style sac of *N. sulcata* were investigated and compared with the composition of the stomach juice. For each set of experiments the enzyme extracts were prepared in the same way throughout the course of the work, viz.:

Stomach juice. The right valve, labial palps, and ctenidium were removed and the foot slit open to reveal the style sac. Removal of the kidney tissue exposed the right posterior wall of the stomach which was then pierced and the contents removed with a fine pipette. In this way the stomach contents were obtained without damage to the tubules of the digestive diverticula. The pH of the stomach fluid lies between 5·7 and 5·9. The stomach contents of 30 animals were diluted with distilled water and centrifuged at 3,500 r.p.m. for 30 minutes; the clear centrifugate was used as the enzyme extract.

Style sac. Unlike the majority of Lamellibranchia the style sac of *N. sulcata* is easily removed free from surrounding tissue. The style sacs of 30 animals were rinsed a number of times with distilled water to remove any traces of the stomach contents and ground up in a small glass mortar with clean sand and distilled water (for protease experiments 25% glycerol). The mixture was centrifuged at 3,500 r.p.m. for 30 minutes and the clear centrifugate used as the enzyme extract.

Digestive diverticula. As with the style sac, extracts of the diverticula were made by grinding the tissue with clean sand and centrifuging the brown liquid at 3,500 r.p.m. for 30 minutes. The debris was thrown down and the fat formed a cake on the top of the yellow centrifugate used as the enzyme extract.

The activity of the enzymes was determined at 23° C, mixtures of the extract and substrate being buffered with McIlvaine's solutions. Boiled controls were rigidly set up and care was taken to exclude saliva from the tubes; bacterial action was prevented by the addition of toluene. Reducing sugars were estimated by the method of Hagedorn and Jensen (as modified by Boyland, 1928), while protein digestion was investigated by the gelatine plate method of Gates (1927) as modified by Pickford and Dorris (1934), and by the Sørensen formol-tritration method, the formaldehyde being added after neutralization to phenolphthalein. Qualitative tests for lipase were made by mixing the extracts with an emulsion of olive oil and subsequently staining a small sample with Nile blue sulphate (George, 1952). The duration of the amylase experiments, with 1% soluble starch as the substrate, was 32 hours and the extracts from all three sources, the digestive diverticula, the style sac, and the stomach juice, gave positive results. The optimum pH for the activity of the enzyme from the style sac is about 6·2, that of the stomach about 6·0. The pH optimum for the amylase of the digestive diverticula lies between 4·3 and 4·5 with a small but definite increase in activity on the less acid side between 6·1 and 6·4. While the amylase of the style sac is undoubtedly secreted into the stomach cavity, the contribution of the digestive diverticula to the stomach juice is less certain. The data obtained from experiments on extracts of the digestive diverticula seem to indicate the occurrence of two enzymes, one having a distinct optimum at pH 4·4 and the other an indistinct one at pH 6·2

The determination of pH optima is not a very safe means for the identification of enzymes, but a comparison of the pH optima of extracts of the digestive diverticula with the stomach juice suggests that the main amylase occurring in the diverticula is not discharged into the stomach but acts intracellularly, while the second and weaker amylase, having a pH optimum between 6·1 and 6·4, is secreted into the stomach. It is interesting to note that the optimum pH of the amylase of the diverticula is lower than that usually recorded for animal amylase (pH 5·5–7·0), although B-amylase, traces of which occur in the crop juice of *Helix pomatia* (Anker and Vonk, 1946), has an optimum pH of about 4·5.

A weak protease capable of attacking gelatine and casein was identified in the stomach juice and in extracts of the digestive diverticula; extracts of the style sac gave negative results. The protease in the stomach juice is probably secreted by the digestive diverticula, since both extracts were most active between 5·8 and 6·6. Under the conditions of the experiment sharp optima were not obtained.

Tests for lipase activity were positive in extracts of the digestive diverticula, of the style sac, and of the stomach juice. These results are interesting since George (1952), using a similar method, demonstrated the presence of a lipase in the crystalline styles of members of the Anisomyaria and Eulamellibranchia.

The Probable Functioning of the Stomach and Digestive Diverticula

The method of feeding of the Nuculidae has been described by Hirasaka (1927) and Yonge (1939). From the observations given above, the working of the alimentary canal may be summarized as follows. The particulate matter forming the bottom deposits, and including sand grains, living organisms, and organic detritus, is collected by the palp proboscides and ingested in considerable quantity, so that in freshly caught animals the stomach is invariably distended. The presence of a sphincter muscle at the junction of the style sac with the intestine prevents the escape of material from the stomach except by the intestinal groove. Enzymes are secreted into the lumen by the style sac epithelium and by the digestive diverticula, and food and enzymes are thoroughly mixed by the rotating action of the specialized cilia of the style sac. The pressure exerted by the distended wall of the stomach, aided by muscular action, serves to squeeze the soluble products of digestion out of the contained mass and these are absorbed by the lining epithelium. Finally, relaxation of the sphincter muscle allows the compacted faecal mass contained in the style sac to enter the intestine.

The ciliary currents over the major and minor typhlosoles and of the sorting areas of the anterior region of the stomach play only a minor part in the circulation of material within the stomach. The most actively beating cilia in the stomach are those surrounding the slit-like apertures to the ducts of the digestive diverticula. They are of considerable length and presumably serve to drive fluid out of the ducts, while at the same time preventing material from

entering the diverticula. This was frequently demonstrated in sections passing through the junction of the ducts with the stomach. The lumen of the stomach was filled with material, while that of the ducts within the ciliated ridges surrounding the apertures was completely devoid of all solid material other than the various granules derived from the tissues of the digestive diverticula. The ciliary currents over the major and minor typhlosoles, the crests of the folds of the sorting areas, and the various ridges of the stomach are for the most part directed dorsally, while those of the various grooves are directed ventrally and drain into the intestinal groove. In the dissected stomach the dorsally directed currents are very weak and capable of moving only the finest particles. They probably serve to drive fluid dorsally away from the intestine, while those of the grooves direct fine particles into the intestinal groove and so to the intestine.

There seems little doubt that the darkly staining flagellated cells of the tubules are secretory, the undulating motion of the flagella driving the secretion away from the narrow distal regions of the cells, but the function of the granule cells remains obscure. During the fragmentation phase, the cells are tall with the tips rounded and club-shaped (fig. 6, A). Large spheres, containing both spherical and irregular granules, are freely constricted into the lumen and are conveyed out of the tubules by the cilia of the secondary ducts. The majority break down in the lumen of the main ducts to liberate the granules which are found free in the stomach. It is not certain whether the granules disintegrate in the lumen of the stomach, or retain their identity and pass out with the faecal material.

Alternating with the fragmentation phase, the free surface of the granule cells is flat and entire, and shows a striated appearance suggesting a poorly developed brush-border (fig. 6, B). Large clear vacuoles with a slightly granular content occupy the distal region of the cells, while the middle and basal regions are filled with irregular and spherical granules respectively. The general appearance of the cells is very similar to that of the absorptive phase in the corresponding cells of other molluscs, but in the Nuculidae there is never any evidence of absorption. It is possible that in these animals this represents a secretory phase.

Discussion

Although the Nuculidae are in certain respects the most primitive living lamellibranchs, the structure of the stomach and digestive diverticula suggests a considerable degree of specialization, probably correlated with what is perhaps the most interesting feature of the alimentary canal in these animals—the complete absence of intracellular digestion.

The stomach

As suggested by Morton (1953) the stomach of the primitive mollusc probably consisted of (*a*) a ciliary sorting area converging on the intestinal groove; (*b*) an area of cuticle forming the gastric shield; (*c*) a style sac connecting the stomach with the intestine and lined with strong cilia beating trans-

rsly (fig. 8, A). Graham (1949) suggested that the primitive stomach also ossessed a caecum into which the major typhlosole extended. A feature of the omach of the Nuculidae when compared with the primitive mollusc is a eneral simplification of structure, presumably correlated with the loss of tracellular digestion. This is illustrated by a reduction in importance of that art of the major typhlosole which extends across the floor of the stomach. It as been suggested (Graham, 1949) that in the primitive mollusc the major phlosole extended across the floor of the stomach to end at the tip of a ouch-like caecum (fig. 8, A). The intestinal groove continued round the tip of he typhlosole and emerged from the caecum to end at the opening of the ft duct of the digestive diverticula. Moreover, the typhlosole was probably flap-like structure which arched over the intestinal groove and isolated the aste products derived from the digestive diverticula from the general circulaon of material within the gastric cavity. The flap-like nature of the major phlosole is well developed in both the Anisomyaria and Eulamellibranchia, nd in the latter (fig. 8, B) it extends into the right and left caeca to act as a alve controlling the entry of material into the diverticula (Owen, 1955). hese features, which are undoubtedly correlated with the function of the igestive diverticula as organs of intracellular digestion, are not shown by the tomach of the Nuculidae. The extension of the major typhlosole across the oor of the stomach is represented by a poorly developed fold which does not nter the relatively small caecum (fig. 8, C). This trend towards a general reuction in the importance of the major typhlosole and of the caecum also ccurs in the Gastropoda (Graham, 1949) and, as in the Nuculidae, is ssociated with increased extracellular digestion.

As in the Anisomyaria and Eulamellibranchia, the stomach of the Nuculidae ossesses extensive sorting areas, a feature normally associated with intraellular digestion. In the Nuculidae, however, the ciliary currents over these egions are so weak as to have little effect on the relatively coarse material resent in the stomach. It has been suggested that the stomach of the Nucudae is capable of extensive trituration of the ingested particles, which may ttain a diameter of over 100μ. A comparison of the contents of the stomach ith those of the intestine shows an increase in the amount of finely divided aterial present in the latter, but the maximum particle-size is the same in oth. The most striking difference in the contents of the intestine when comared with those of the stomach, is the increase in the number of more or less omplete but empty diatom tests and protozoan skeletons. This suggests that hile some mechanical trituration occurs in the stomach, enzymes are also resent and, as already suggested, the weak ciliary currents of the sorting reas may serve to separate from the soluble products of digestion the fine articles which escape from the central mucous mass.

he style sac

An interesting feature of the stomach of the Nuculidae is the secretion of nzymes by the epithelium of the style sac although a firm rod-like crystalline

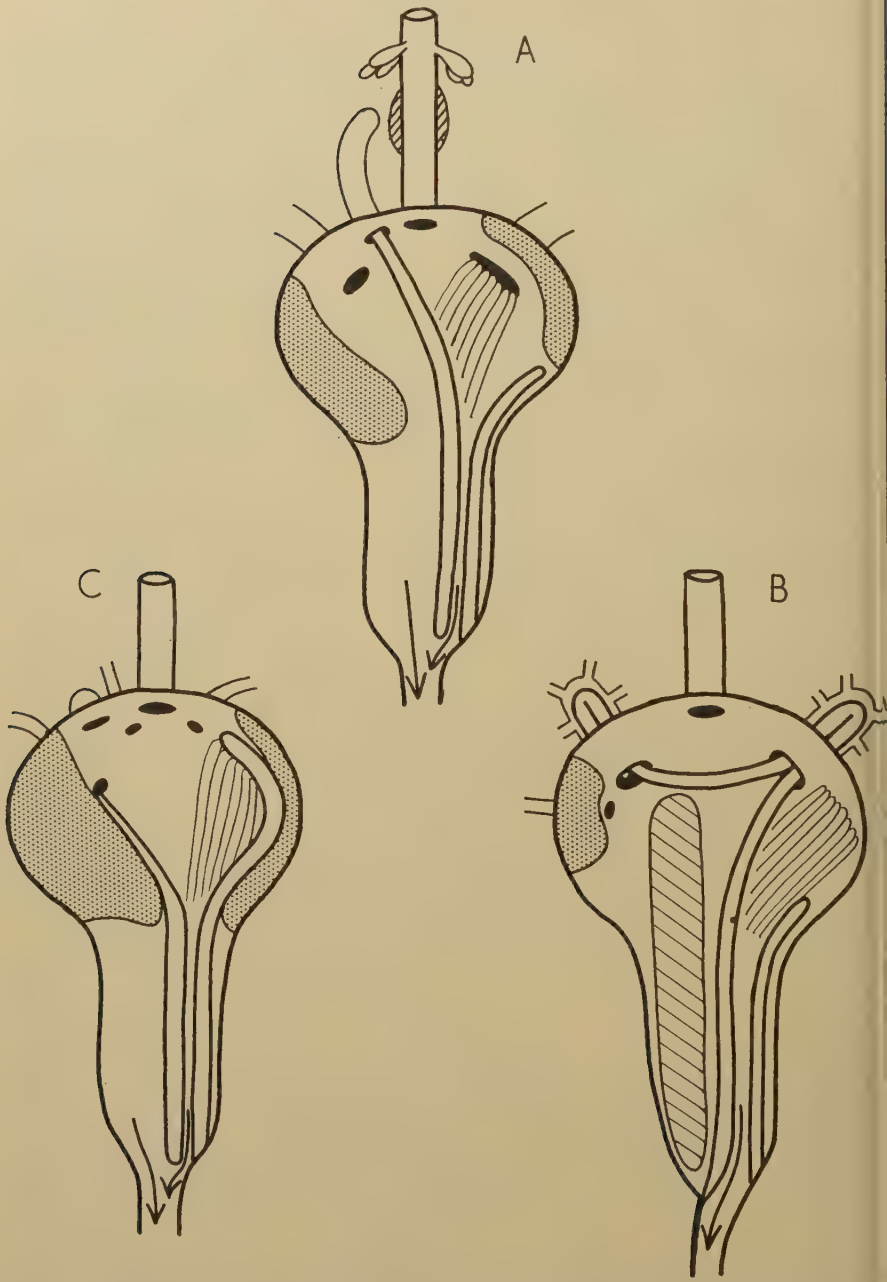


FIG. 8. Diagrammatic representations of the stomachs of A, the primitive mollusc (afte
Graham, 1949); B, a eulamellibranch; C, *Nucula*.

yle is never developed. Much has been written upon the crystalline style in e Mollusca, but the above observation is of sufficient interest to warrant rther discussion on this subject. Most workers are agreed (see Yonge, 1939; raham, 1949; Morton, 1952) that the relatively loose mucus-bound mass ling the style sac in the Nuculidae is homologous with the firmer rod-like ructure of the Anisomyaria and Eulamellibranchia. This is supported by the nilarity of the style sac epithelium in all three groups. Yonge (1939), raham (1949), and Morton (1952) all consider the style sac of the primitive ollusc and its contained protostyle to have been concerned in the first place ith purely mechanical functions, and the presence of enzymes a specialized haracter which subsequently evolved independently in both the Gastropoda nd Lamellibranchia. Morton (1952) defines the protostyle as not containing ny enzymes.

In a comparison of the various members of the protobranchiate mollusca, onge (1939) concluded that the Nuculidae are the least specialized and pos-bly represent the most primitive living Lamellibranchia. It is of interest, herefore, to compare the conditions now known to exist in the Nuculidae ith those which are thought to characterize the primitive protostyle. In oth, the style sac serves to consolidate faecal material and aids in the forma-on of a faecal rod which is periodically released into the intestine by the elaxation of a sphincter muscle. The stiff, bristle-like cilia of the epithelium f the style sac rotate the contained mass and so mix the contents of the tomach. These appear to be the only features common to both the Nuculidae nd the primitive mollusc as defined by Morton (1952). The main difference etween the style sac of the Nuculidae and that of the primitive mollusc is the ecretion in the Nuculidae of amylolytic and lipolytic enzymes by the style ac epithelium. In view of this difference, it is interesting to speculate how far onditions in the Nuculidae may be regarded as primitive. Morton's (1952) efinition of the protostyle as not containing any enzymes is based on the bsence of enzymes from the protostyle of primitive gastropods and the ondition then thought to exist in the Nuculidae. Whether enzymes are bsent from the corresponding region of the gut of the Loricata is not known. Thus, the suggestion that the primitive protostyle did not contain enzymes is ow based solely on conditions existing in the primitive gastropods. Never-heless, until the Loricata and, if possible, primitive members of the archaeo-astropods are re-investigated, this must be accepted although it is difficult to isualize the same region of the gut evolving independently the ability to ecrete enzymes in both the Gastropoda and the Lamellibranchia.

The well-developed style sac of the Nuculidae suggests that the absence of a irm rod-like crystalline style may be a secondary condition, possibly related o the presence of an extracellular protease (Yonge, 1930), but this is unlikely. The Nuculidae feed by means of the labial palps and in particular the palp roboscides, the ctenidia serving only a minor role in the collection of food (Hirasaka, 1927). Thus members of the Nuculidae are essentially detritus eeders and consequently ingest, together with organic 'food', large quantities

of inorganic material. Enzymes are secreted into the lumen of the stomach and mixed with the contained mass by the rotating action of the cilia of the style sac and the soluble products of digestion are squeezed out of the faecal material. Such a method of extraction would be difficult, if not impossible, in a stomach containing a firm rod-like crystalline style. Furthermore, the remaining faecal mass, which constitutes the bulk of the ingested material, is periodically released into the intestine, the style sac forming a through passageway between the stomach and intestine (fig. 8, A and C). In the Anisomyaria and Eulamellibranchia, the firm rod-like crystalline style is no longer faecal and all material entering the intestine does so by way of the intestinal groove alone. This feature is probably correlated with the finely divided nature of the food and is an essential preliminary to the development of a firm, non-faecal crystalline style (fig. 8, B). Yonge (1939) suggested that the development of labial palps as exemplified by the Nuculidae illustrates the transition from the primitive mollusc, having the mouth in contact with the substrate and feeding with the aid of the radula, to the suspension-feeding Anisomyaria and Eulamellibranchia where the greatly enlarged and complex ctenidia remove particulate material from the inhalant current. It follows that the Nuculidae have not evolved from suspension-feeding ancestors and it is unlikely that they ever possessed a firm crystalline style, the failure to develop such a style being correlated with the method of feeding and the functioning of the stomach. The relatively large size of the stomach and style sac is presumably an adaptation for the ingestion of large quantities of bottom material.

The digestive diverticula

Although much work has been done on the digestive diverticula of the Lamellibranchia, attention has been largely restricted to the structure and function of the blindly ending tubules. It is not surprising, therefore, that the considerable morphological differences which exist between the diverticula of the Nuculidae and those of the Anisomyaria and Eulamellibranchia have been overlooked, since they are most marked in the structure of the ducts leading from the tubules to the stomach (fig. 9, C and D). In the Anisomyaria and Eulamellibranchia (fig. 9, C), the main ducts are ciliated with the ciliated cells restricted to a well-defined gutter; the short secondary ducts are non-ciliated and the lumen surrounded by a brush-border epithelium (Owen, 1955). In the Nuculidae, on the other hand (fig. 9, D), it is the main ducts which are non-ciliated while the cells lining the secondary ducts possess well-developed long cilia which extend more or less to the middle of the lumen.

Despite the different results obtained from feeding experiments, the general appearance of the tubules of the Nuculidae is very similar to that of the Anisomyaria and Eulamellibranchia. In all three groups the epithelium is divided into two regions, one composed of numerous lightly staining cells and the other of smaller and less frequent darkly staining, flagellated cells. Yonge (1926) concluded that the epithelium was composed of one type of cell only, the darkly staining cells being nests of young cells which by dividing

eplace the older lightly staining cells. This does not appear to be the case in he Nuculidae. Intermediate stages between the two types were never observed, and in these bivalves the darkly staining cells are probably secretory nd excretory. In the Nuculidae the appearance of the contents of the granule ells differs markedly in sections from those of the corresponding cells in the Anisomyaria and Eulamellibranchia. In the latter after most fixatives and tains, these cells normally present an extremely vacuolated appearance, while feeding experiments with iron saccharate, colloidal graphite, and titanum dioxide demonstrate convincingly their absorptive function and their ability to ingest solid particles; the ingested material is 'concentrated' in the vacuoles (Owen, 1955). In the Nuculidae, on the other hand, the middle and basal regions of these cells are filled with densely staining granules. There is no ingestion of solid particles nor any evidence of absorption, the appearance of iron in the granules being probably due to transport in the blood after absorption by the stomach epithelium. Moreover, it is difficult to visualize how an interchange of fluid between the lumen of the stomach and of the blindly ending tubules could take place. Cilia are restricted to the relatively short and unbranched secondary ducts and beat away from the tubules, the longer and much-branched main ducts being non-ciliated with their slit-like openings into the stomach surrounded by long cilia beating actively into the stomach (fig. 9, D).

In the Lamellibranchia, the structure and functioning of the digestive diverticula are correlated with the nature of the ingested food. The primitive mollusc presumably possessed a well-developed head, and fed with the aid of the radula on living algae and possibly detritus, and on the available evidence digestion must have been in part intracellular. Despite the triturating action of the radula, the ingested food almost certainly included relatively large fragments, and material present in the stomach was probably pumped from and into large, simple, sac-like diverticula by muscular activity, there being practically no duct system (fig. 9, A). Within the diverticula, solid particles were ingested by the cells for the completion of digestion intracellularly in a manner similar to that which takes place in the wide diverticula of the Nuculanidae (Yonge, 1939). The structure and mode of functioning of the digestive diverticula in the larval oyster are similar. Particles are drawn into the diverticula and returned to the stomach by the rhythmic expansion and contraction of the diverticula. Each diverticulum is simple and sac-like and opens more or less directly into the stomach (Millar, 1955). In the septibranchs, *Cuspidaria* and *Poromya* (Yonge, 1928), relatively large particles enter the tubules of the diverticula probably as a result of pumping movements by the muscular stomach, but again the ducts are exceptionally short and the tubules relatively large, simple sac-like structures (fig. 9, B).

Intracellular digestion is also retained by the Anisomyaria and Eulamellibranchia, but the area of the ingesting cells is greatly increased in the majority of adults by the development of numerous small tubules served by an extensive and much-branched duct system (fig. 9, C). Muscular activity plays little

part in the circulation of material, and is probably restricted to the extrusion of waste material from the tubules into the main ducts. The main circulation of fluid and particles within the diverticula is maintained by the specialized tracts of cilia of the main ducts (Owen, 1955). The majority of the Anisomyaria and Eulamellibranchia are suspension feeders, and the ciliary mechanisms of the stomach and ducts of the diverticula are admirably adapted for dealing rapidly and continuously with numerous fine particles. Thus muscular

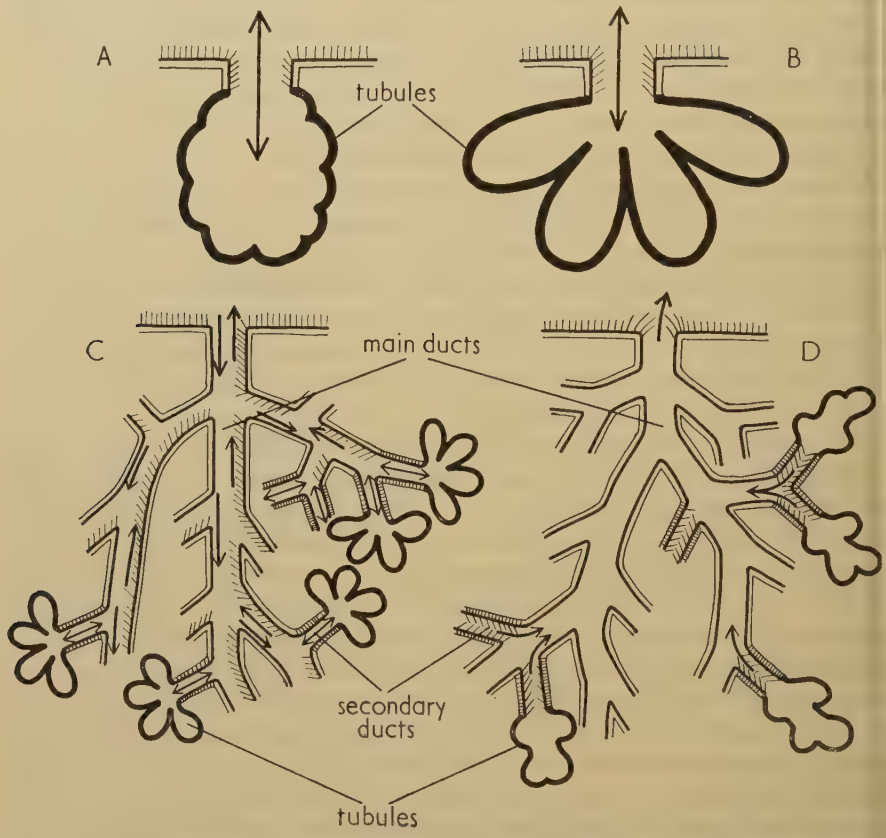


FIG. 9. Types of digestive diverticula present in the Lamellibranchia, shown diagrammatically. A, hypothetical primitive condition and also many larval lamellibranchs; B, septibranchs, some eulamellibranchs, and the wide diverticula of the Nuculanidae; C, Anisomyaria and most Eulamellibranchia; D, Nuculidae. Double-headed arrows represent movement resulting from muscular activity; single-headed arrows represent ciliary currents.

pumping movements as the sole means of controlling the passage of material from and into the diverticula appear to function satisfactorily only in those lamellibranchs in which the duct system is poorly developed and the tubules relatively simple sac-like structures opening more or less directly into the stomach. The diverticula of the Nuculidae also possess an extensive and much-branched system of ducts and tubules (fig. 9, D), and it is unlikely that muscular activity could result in an efficient interchange of fluid. Muscle

fibres round the ducts and tubules are poorly developed and pumping movements by the stomach would almost certainly result in solid particles, in addition to fluid, entering the diverticula. This was never observed.

Thus, although the general appearance of the digestive diverticula of the Nuculidae is similar to those of the Anisomyaria and Eulamellibranchia, they are not organs of intracellular digestion or even of absorption. They are excretory and secretory in nature, and specialized tracts of cilia controlling a two-way flow of fluid and particles are not developed (fig. 9, D). Digestion is entirely extracellular and the soluble products are absorbed by the epithelium of the stomach and the first part of the intestine. This loss of the primitive method of intracellular digestion is presumably correlated with the method of feeding. The Nuculidae are detritus-feeders and the ingested material includes both large particles and a high proportion of inorganic material. Under such conditions the retention of intracellular digestion would result in considerable waste, since the cells of the digestive diverticula would ingest relatively large quantities of inorganic material. (That the cells of the diverticula of other lamellibranchs will ingest inorganic particles is demonstrated by feeding experiments with titanium dioxide (Owen, 1955) and the observation of Yonge (1939) on *Yoldia* that the wide diverticula 'were almost black in colour owing to the presence in the cells and in the lumina of numerous particles of black sand'.) This loss of intracellular digestion and adoption of extracellular digestion, although surprising in primitive members of the Mollusca, is not unusual, a similar change having taken place on a number of occasions in the Gastropoda. It is remarkable, however, that, as in the Cephalopoda, the digestive diverticula have also lost their absorptive function. While the Nuculidae are in many respects primitive lamellibranchs and in their method of feeding occupy an intermediate position between the radula-feeding primitive mollusc and the suspension-feeding Anisomyaria and Eulamellibranchia, the structure and physiology of the gut present many specialized features. It is suggested that this specialization is correlated with the retention of the labial palps as feeding organs and the ingestion of large quantities of inorganic material with the food.

The ciliation of the tubules

A minor problem presented by the digestive diverticula of the Mollusca, at least in the Loricata, Gastropoda, and Lamellibranchia, is the ciliation of the tubules. From observations on fresh preparations of the diverticula of various lamellibranchs, Potts (1923) described cilia beating with a rather languid motion within the tubules. He was unable to demonstrate their presence in sections of fixed material and concluded that the cilia were retractile. Similar observations on the presence of cilia within the tubules in fresh preparations of the diverticula of a variety of chitons, gastropods, and lamellibranchs have been made by many workers, but in all cases cilia were not seen in sections of fixed material. There have been various explanations: that the cilia are retractile, that they drop off on fixation, that the tubule cells

pass through phases and that the striated appearance frequently presented by them represents the remains of cilia. In the Nuculidae, Anisomyaria, and Eulamellibranchia, the cilia (termed in this paper 'flagella') are borne by the darkly staining cells of the tubules. In the Eulamellibranchia there are a small number of flagella to each cell and consequently they are relatively easily seen in sections of fixed material (Owen, 1955). In the Nuculidae they are more difficult to observe both in fresh material and in sections, there being but a single flagellum to each cell (fig. 6). Flagella are also borne by the darkly staining cells of the Anisomyaria. The number per cell is not known, although it is certainly less than in the Eulamellibranchia and there may be only one per cell as in the Nuculidae. It seems likely that flagella are always present in the tubules of chitons, gastropods, and lamellibranchs, and the difficulty experienced in demonstrating them in sections of fixed material is due to their distribution, e.g. one per cell as in the Nuculidae, rather than to their being retractile or cast off as a result of fixation.

As in previous papers, it is a pleasure to record my indebtedness to Professor C. M. Yonge, C.B.E., F.R.S., for his kindness and helpful criticism. I am also indebted to the Director and Staff of the Millport Laboratory for the supply of living material.

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The Use of the Baker Interference Microscope for the Study of Optically Heterogeneous Specimens

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Summary

The refractive index of an optically heterogeneous specimen will generally vary in directions parallel and normal to the microscope axis, but a mean index can be defined such that the optical thickness of the specimen is equal to the product of the mean index and the specimen thickness. If such a specimen is examined under an interference microscope, the fringe displacement will be zero at those parts of the specimen for which the mean index is equal to the index of the immersion liquid. Thus, by gradually changing the index of the liquid relative to that of the specimen, one can determine the manner in which the mean index varies from region to region of the specimen. In the present work these changes in relative index have been effected by varying either the wavelength of the incident light or the temperature of the system.

The Baker two-beam interference microscope has been successfully used for such investigations. Three types of interference pattern have been tried: (i) a single fringe is made to fill the field of view and the intensity differences between the specimen and the background are studied; (ii) monochromatic fringes are formed in a quartz wedge and their linear displacements are examined; and (iii) a quartz plate is used, channelled spectra being produced by the spectrographic analysis of the interference colours. The first two types can be used with either the temperature or the wavelength variation method, but the third was specifically designed for use with the wavelength variation method. Although these experiments have mostly been carried out on birefringent fibres, the techniques are applicable to any kind of object. The attainable accuracy increases with specimen thickness; under favourable circumstances the index at any point of a specimen 10 μ thick should not be in error by more than $\pm 3 \times 10^{-4}$.

Introduction

THE writer has recently described the way in which multiple-beam interference microscopy can be used in order to determine the refractive index variations within optically heterogeneous specimens (Faust, 1952, 1954). The aim of the present paper is to show how the same kind of information can be obtained with the Baker two-beam interference microscope (Smith, 1950, 1955). Although the emphasis is upon the examination of birefringent textile fibres, the methods described are applicable to any kind of object.

If a solid of index μ_S is immersed in a liquid of index μ_L, the phase difference ϕ introduced by the object is given by the expression

$$\phi = (2\pi/\lambda)(\mu_S - \mu_L)t = 2N\pi, \qquad (1)$$

where t is the thickness of the solid, λ the vacuum wavelength of the light, and N is a real number, not necessarily integral. If this specimen is examined under an interference microscope, any changes in ϕ will be observed as changes in the displacements of the interference fringes. In particular, there will be no fringe displacement whenever ϕ is zero, that is, whenever the index of the liquid is equal to that of the solid.

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The importance of interference microscopy lies in its application to th study of optically heterogeneous specimens. For instance, Frederikse (1934 1935) has employed multiple-beam interference in monochromatic light i order to study the structure of animal cells. Since he was unaware of th variations in the cell thickness t, he compressed the cell between the inter ferometer plates so that all parts were of the same thickness. Fringe displace ments then portrayed changes in the index μ_S from one part of the cell t another. (In optically heterogeneous specimens the refractive index is no necessarily constant along a line parallel to the microscope axis, and μ_S wil therefore represent a mean index so defined that the optical thickness at tha part of the specimen is $\mu_S t$.) Deformation of the specimen is, however, un desirable, and some workers have therefore measured the integral of $(\mu_S-\mu_L)$ over the projected area of the undeformed cell; in this manner they could fin the total dry mass of the cell without knowing the variations in cell thickness (Barer, 1952; Davies and others, 1952, 1954). Although this method migh have limited applications in fields other than that of cytology, it is often mor satisfactory to measure the mean refractive index at different points of th specimen; with birefringent specimens there will be a different mean inde for each vibration direction. From such information conclusions can be draw regarding the molecular orientation and the closeness of molecular packing i different regions of the specimen (Faust, 1952, 1954).

In order to obtain the mean index the specimen can first be immersed in liquid of index μ_L and then in another of index μ'_L. The two resultant inter ference patterns will yield values for $(\mu_S-\mu_L)t$ and $(\mu_S-\mu'_L)t$ respectively, from which it is possible to calculate the mean index μ_S and the thickness t at an desired points of the specimen (Barer, 1953; Davies and others, 1954). Instead of using two different immersion liquids, one can alter either the wavelengt of the incident light or the temperature of the system. In this way it is quit easy to change the refractive index difference $(\mu_S-\mu_L)$ by at least 0·003 and, i this is done in small steps, the procedure is equivalent to the use of a larg number of immersion liquids. This has the advantage of converting th method into a null one: the conditions can be varied until the fringe displace ment at any point of the specimen becomes zero, whereupon it is concluded that the mean index of the solid at that point is equal to the index of th liquid. A further advantage is that the specimen is not disturbed during th experiment and, consequently, the same part of the specimen is always pre sented to the observer in exactly the same manner. Both the temperature an wavelength variation methods have been successfully used with multiple beam interference microscopy, and it was therefore decided to apply thes same methods to the Baker interference microscope.

The Baker Interference Microscope

In the Baker interference microscope two coherent wavefronts are produce by the use of birefringent components in both the condenser and the objective A shearing and a double-focus system are in commercial production.

In the shearing system the incident light passes through a polarizer before entering the condenser, where it is split into two beams, the extraordinary W_1 and the ordinary W_2. These beams, polarized at right angles to one another, suffer a relative lateral displacement, this effect being represented diagrammatically in fig. 1. Immediately after the condenser is a half-wave plate which rotates the planes of polarization through 90° so that the extraordinary beam W_1 from the condenser becomes the ordinary beam in the objective, and conversely. (The optic axes of the calcite plates in the condenser and objective are

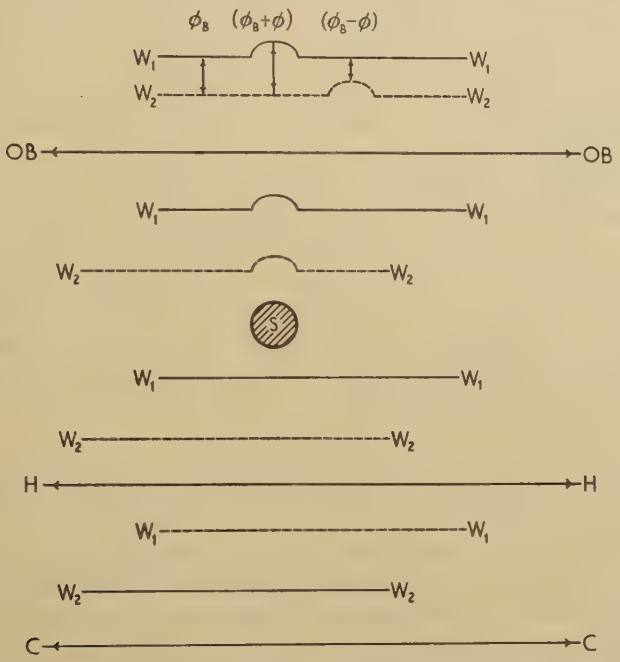

FIG. 1. The Baker shearing system. *CC* condenser, *HH* half-wave plate, *OB* objective, *S* isotropic specimen. W_1 and W_2 represent the wavefronts, a dashed line indicating that the electric vector is in the plane of the paper, a continuous line that it is normal to the paper.

parallel.) This arrangement not only removes the lateral displacement but ensures that, in the absence of the specimen, the two emergent beams possess similar ellipsoidal wavefronts. The two beams are, however, still polarized at right angles to one another and interference effects can be obtained only by the introduction of an analyser.

The lateral displacement of the two beams is such that, in the presence of a specimen, two images are seen in the field of view. The extraordinary image, that is the image formed by the extraordinary beam W_2 from the objective, is uncorrected for astigmatism, and the ordinary image should therefore be used whenever possible. (Mr. F. H. Smith has suggested that a cylindrical lens could be designed which, when placed over the eyepiece, would correct the astigmatism of the extraordinary image. This lens would be removed

whenever the ordinary image was being studied. The present paper describes the procedure to be followed when such a lens is not available.)

With the double-focus system the ordinary and extraordinary beams from the condenser do not suffer any lateral displacement but are brought to a focus at different distances from the condenser. One beam is focused on the plane containing the specimen and its wavefront is thereby deformed. Since the other beam must act as a reference wave, it is essential that it should be focused on a plane other than that containing the specimen and that a condenser with a large numerical aperture should be used; under such conditions only a small fraction of this beam will pass through the specimen. As with the shearing system the roles of ordinary and extraordinary are reversed in the objective and once again an analyser is necessary.

The shearing system can be used with a restricted condenser aperture and, unlike the double-focus system, is therefore satisfactory for the examination of highly birefringent specimens. Another advantage of the shearing system lies in the fact that relatively large features can be studied: with the $\times 10$, $\times 40$ and $\times 100$ shearing systems the lateral displacements of the two beams are 330, 160, and 27 μ respectively, whereas with the corresponding double-focus objectives the diameters of the reference areas are 90, 90, and 20 μ respectively. Hence, in the following pages reference is made only to the shearing system, but it must be remembered that the same basic principles can be applied equally well to the alternative system.

Examination of Birefringent Specimens

The vibration directions of the birefringent components of the condenser are parallel to those of the objective. In fig. 2 these directions are represented by the X and Y axes. The X axis, which corresponds to the vibration direction of the extraordinary beam from the objective, is also the direction of shear. If the specimen is isotropic, its orientation with respect to X and Y is unimportant provided that the width of the specimen in the direction of shear does not exceed the shearing distance. If, however, it is birefringent, its vibration directions must be parallel to X and Y so that the polarization directions of the light will be unaffected by the specimen. For example, if the specimen is a fibre with its vibration directions parallel ($\parallel$) and perpendicular ($\perp$) to the fibre axis, the fibre orientation should be as in fig. 2, A (Y setting) or fig. 2, B (X setting).

In the background part of the field of view the phase difference ϕ_B between the two waves W_1 and W_2 will depend upon the experimental arrangement; only when the wavefronts W_1 and W_2 are parallel will ϕ_B be the same for all parts of the field. In those parts of the field containing the images of the fibre the phase difference between the two waves is $(\phi_B+\phi)$. For the Y setting the appropriate values of ϕ are:

ordinary image $\quad \phi = \phi_{\parallel} = (2\pi/\lambda)(\mu_{\parallel}-\mu_L)t$; (2a)

extraordinary image $\quad \phi = -\phi_{\perp} = -(2\pi/\lambda)(\mu_{\perp}-\mu_L)t$. (2b)

The extraordinary image suffers from astigmatism, but, provided that the structure under examination is clearly resolved, the information derived from this image will be reliable. In order to obtain a non-astigmatic image for vibrations at right angles to the fibre axis, the X setting should be used. A piece of fibre, of length equal to the shearing distance, will then be portrayed by the ordinary image and equation (3a) will be applicable. The remainder of the fibre will be portrayed by the overlapping ordinary and extraordinary

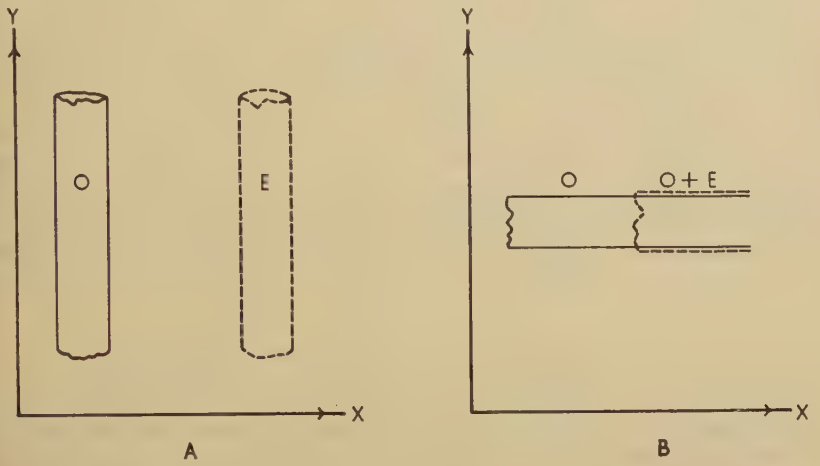

FIG. 2. The image positions in (A) the Y setting, and (B) the X setting. The X-axis represents the vibration direction of the extraordinary beam from the objective. The full line represents the ordinary image, the dashed one the extraordinary image. If a wedge is used, the line of greatest slope is parallel to Y in (A) and to X in (B).

images; if the properties of the fibre are constant along the fibre length the appropriate value of ϕ will be given by equation (3b):

ordinary image $\phi = \phi_\perp = (2\pi/\lambda)(\mu_\perp - \mu_I)t$; (3a)

overlapping images $\phi = (\phi_\perp - \phi_\parallel) = (2\pi/\lambda)(\mu_\perp - \mu_\parallel)t$. (3b)

The interference pattern observed in the overlap region will therefore be dependent upon the birefringent path difference. Measurements of the observed fringe displacements will not, however, furnish precise values for this path difference because of the astigmatism of the extraordinary image and because both images are not simultaneously in sharp focus.

If the fibre is so oriented that its vibration directions are not parallel to X and Y but are inclined at an angle α to them, the measured phase difference is no longer $\phi_\parallel$ or $\phi_\perp$ as defined above. This is evident from the fact that the phase difference determined from the ordinary image changes from $\phi_\parallel$ to $\phi_\perp$ as the angle α between the fibre axis ($\parallel$ vibration direction) and Y increases from 0 to $\frac{1}{2}\pi$. However, it is shown below (p. 588) that, if α is small, the ordinary image yields the phase difference $(\phi_\parallel + \chi)$, where

$$\chi = \alpha^2 \sin(\phi_\perp - \phi_\parallel). \quad (4)$$

Thus, even if α is as large as 0·1 radian, χ is at the most only $\pm$0·01 radians corresponding to a path difference of $\pm\lambda/600$. Such a small systematic error is negligible, since it is less than the sensitivity of the instrument. It should not however, be concluded that accurate orientation of the fibre in either the X or Y setting is unimportant. Whenever α has a value other than 0 or $\frac{1}{2}\pi$, the contrast of the interference pattern suffers, the minimum intensity no longer being zero. If α is small, the ratio of minimum to maximum intensity is given by $[\alpha \sin \frac{1}{2}(\phi_\perp - \phi_\parallel)]^2$. For an angle α of 0·1 radian, this ratio could be as high as 0·01, and the determination of the position of minimum intensity would then be more difficult. This source of uncertainty is most important in the method involving the comparison of intensities.

These considerations show that precise phase measurements are attained only when the fibre is accurately oriented; for this purpose a rotating stage is essential. Specimens in which the vibration directions are variable (e.g. spherulitic structures in nylon) obviously present difficulties. In the following sections it is therefore assumed that the vibration directions of the fibre lie along X and Y.

Temperature and Wavelength Variation Methods

In this section the underlying principles of the variation methods are discussed. The symbol ϕ, representing the phase difference introduced by the specimen, will denote either $\phi_\parallel$ or $\phi_\perp$ in accordance with the considerations of the previous section.

Temperature variation

In a preliminary experiment an immersion liquid is found with a refractive index close to that of the solid; for this purpose the Becke line method can often be used. The immersed solid is then examined under the interference microscope in monochromatic light. At the beginning of the experiment the specimen is at the temperature θ_0 and, if the immersion liquid has been carefully chosen, the interference pattern will indicate that at the point P_0 of the solid the phase difference is zero; that is, $(\mu_S)_{\theta_0} = (\mu_L)_{\theta_0}$ at P_0. Similarly, at another temperature θ a point P will be found for which $(\mu_S)_\theta = (\mu_L)_\theta$, and so on for other temperatures. Hence, the index at P at the temperature θ_0 is

$$(\mu_S)_{\theta_0} = (\mu_L)_{\theta_0} + (\mu_S - \mu_L)_{\theta_0} - (\mu_S - \mu_L)_\theta. \qquad (5$$

In order, therefore, to find the refractive index at different points P_0, P, . . . of the solid at the same temperature θ_0, one must know the temperature difference $(\theta - \theta_0)$, the index $(\mu_L)_{\theta_0}$, and the difference between the temperature coefficients of refractive index of the liquid and the solid.

The temperature coefficient of the liquid can be readily determined on a suitable refractometer. In order to determine the temperature coefficient of the solid one may use a series of liquids with known refractive indices and temperature coefficients and note the temperature at which the index of each liquid equals that of the solid. Although the Becke line method can often b

used to detect when the indices are equal, this method must be regarded as unreliable whenever the optical heterogeneity of the solid is marked (Faust, 1955, 1956). It might then be preferable to employ interference microscopy; the same part of the solid should be examined in each liquid and a note made of the temperature at which the fringe displacement disappears. Fortunately it is not always essential to know the temperature coefficient of the solid accurately as its value is often only about one-tenth that of the temperature coefficient of the liquid.

Undoubtedly the most elegant way of finding the unknown quantities in equation (5) is to utilize the fringe pattern itself. Suppose that at some point Q of the solid the phase differences, as determined from the fringe pattern, are ϕ'_{θ_0} and ϕ'_θ at the temperature θ_θ and θ. Then

$$(\lambda/2\pi)(\phi'_{\theta_0}-\phi'_\theta) = [(\mu'_S-\mu_L)_{\theta_0}-(\mu'_S-\mu_L)_\theta]t', \tag{6}$$

where the dash notation denotes values at the point Q. It is reasonable to assume that the temperature coefficient of the index of the solid is the same for all parts of the solid, and equations (5) and (6) then lead to

$$(\mu_S)_{\theta_0} \text{ at } P = (\mu_L)_{\theta_0}+(\lambda/2\pi t')(\phi'_{\theta_0}-\phi'_\theta). \tag{7}$$

Hence, if t' and $(\mu_L)_{\theta_0}$ are known, only phase measurements are required in order to find $(\mu_S)_{\theta_0}$ at any point of the solid. In this way measurements of temperature and temperature coefficients are rendered unnecessary. On the other hand, the temperature coefficient of refractive index of the solid can be calculated from equation (6), provided that the temperature of the system and the temperature coefficient of the liquid are already known. If the specimen is of simple geometrical form, there will normally be no difficulty in selecting a point Q of known thickness. If the specimen shape is irregular, it might be necessary to place a standard object (e.g. a right cylinder) alongside the specimen. This standard should be of similar material to the specimen so that the measurements involved in equation (6) can be carried out on the standard (Faust, 1954).

Wavelength variation

This method involves the use of light of different wavelengths, the temperature being maintained constant. With a suitably chosen immersion liquid the points $P_0, P, \ldots$ of the specimen can be found for which the phase difference is zero; that is $(\mu_S)_\lambda = (\mu_L)_\lambda$ at P. Hence, the index at P at the wavelength λ_0 is

$$(\mu_S)_{\lambda_0} = (\mu_L)_{\lambda_0}+(\mu_S-\mu_L)_{\lambda_0}-(\mu_S-\mu_L)_\lambda. \tag{8}$$

In order, therefore, to find the index at different points $P_0, P, \ldots$ of the solid at the same wavelength λ_0, one must know the wavelength difference $(\lambda-\lambda_0)$, the index $(\mu_L)_{\lambda_0}$, and the difference between the wavelength dispersions of the liquid and the solid. These wavelength dispersions may be determined by methods similar to those used for the temperature coefficients (Frey-Wyssling and Wuhrmann, 1939; Faust, 1952, 1954). However, a simplification is again

possible if there is a point Q, either on the specimen or on a standard object o like material, at which the thickness is known. The expression

$$\frac{1}{2\pi}(\lambda_0 \phi'_{\lambda_0} - \lambda\phi'_\lambda) = [(\mu'_S - \mu_L)_{\lambda_0} - (\mu'_S - \mu_L)_\lambda]t' \qquad (9$$

can then be combined with equation (8) to yield

$$(\mu_S)_{\lambda_0} \text{ at } P = (\mu_L)_{\lambda_0} + \left(\frac{1}{2\pi t'}\right)(\lambda_0\phi'_{\lambda_0} - \lambda\phi'_\lambda). \qquad (10$$

Thus, if t' is known, it is only necessary to make phase measurements at known wavelengths. Furthermore, if the wavelength dispersion of the liquid i known, that of the solid can be calculated from equation (9).

It will therefore be appreciated that, although it is possible to study th optical heterogeneity of a specimen without a knowledge of the specimen dimensions, the task is greatly simplified if the thickness is known at on point of the specimen or at one point of a similar but conveniently shape object. Techniques are described below (pp. 576, 585) by which phas measurements can be made with the Baker microscope. These technique belong to three classes, those involving intensity measurements, those usin wedge fringes, and those using channelled spectra. The first two can b employed with either the temperature or the wavelength variation method but the third one has been designed specifically for use with the wavelengt variation method.

Comparison of Intensities

In this and the following sections some of the points raised have been men tioned in the Instruction Manual issued by Messrs. C. Baker of Holborn. Wher the treatment in this manual is adequate, the reference I.M. will be given.

A quarter-wave plate with vibration directions at 45° to X and Y is intro duced between the objective and the analyser, and the screws that act on th birefringent plate in the condenser are adjusted until one fringe fills the bac focal plane of the objective; that is, until the conoscopic field is uniformly illuminated (I.M.). The birefringent plates of the condenser and objectiv have been so cut that, when this adjustment has been made, the wavefront W_1 and W_2 will be parallel to one another in the orthoscopic field. Hence when the eyepiece is replaced, the background of the image will be of uniform tint in white light or of uniform intensity in monochromatic light, and any phase differences introduced by the fibre will be revealed as differences i tint or intensity between the fibre and the background. For precise wor monochromatic light must be used, and the method therefore resolves itsel into one of intensity comparisons.

The analyser is first rotated until the background is as dark as possible an then rotated until that part of the fibre under study is as dark as possible. Th phase difference introduced by the fibre can then be expressed as

$$\phi = 2M\pi + 2\omega = 2N\pi,$$

where M is an integer and ω is the angular rotation of the analyser (I.M.). If part of the fibre is of minimum intensity at the same analyser setting for which the background intensity is a minimum, then for that part of the fibre N is integral. This is illustrated in fig. 3, A, which shows the ordinary image obtained with an isotropic glass fibre of 0·052 mm diameter; the ×40 shearing system was used with mercury green light. As it was already known that the fibre was a right cylinder of uniform refractive index, N could be determined unambiguously merely by counting the number of black bands from the edge of the fibre; the sign of N was deduced from the fact that the Becke line indicated that the refractive index of the immersion liquid was higher than that of the fibre (I.M.). In general, however, it cannot be assumed that the specimen is of uniform refractive index or of gradually increasing thickness from the edge, and some other method of determining N is required; for this purpose a quartz wedge is often valuable (see p. 579).

It is claimed that under favourable conditions phase differences can be determined with an accuracy of about $\pm 1°$; the use of a half-shade device might lead to an even greater sensitivity (Smith, 1954; I.M.). It should be noted that Goranson and Adams (1933) have shown that if the quarter-wave plate introduces a phase difference of $(90 \pm \delta)$ instead of 90°, the resultant systematic error in any phase measurement will depend upon (*a*) the phase difference ϕ to be measured, and (*b*) the square of δ. For example, if $\delta = 5°$, the maximum error, which occurs when $\phi \simeq 60°$, is only 0·1°. However, if $\delta \neq 0$, the minimum intensity at compensation is not zero and the accuracy of setting is accordingly lowered. Even if $\delta = 1°$ the minimum intensity may well lie above the threshold value of the eye and it is therefore important that δ be kept as small as possible.

Since the quarter-wave plate is selected for use with mercury green light (5,461 Å) the temperature variation method should be carried out at this wavelength. If the wavelength variation method is used, the wavelength range should be so restricted as to keep δ within reasonable limits. For example, if the range is 5,200 to 5,700 Å, the maximum value of δ is about 4°. In order to effect such a wavelength variation a monochromator must be used, the wavelength band passed by the monochromator being as narrow as intensity considerations will permit. If the dispersion D_L (between the F and C hydrogen lines) of the immersion liquid exceeds the dispersion D_S of the solid by 0·0050, this wavelength range will enable one to study a variation in μ_S of 0·0015 (i.e. the index difference $(\mu_S - \mu_L)$ changes by 0·0015). At the same time a monochromator band width of 15 Å will lead to a maximum uncertainty in μ_S of $\pm 0{\cdot}000025$.

Wedge Fringes

The adjustment of the microscope follows the procedure outlined at the beginning of the previous section. The quarter-wave plate and the rotating analyser are then removed, and a Wright eyepiece substituted for the normal ocular. This eyepiece is rotated until the built-in analyser has its vibration

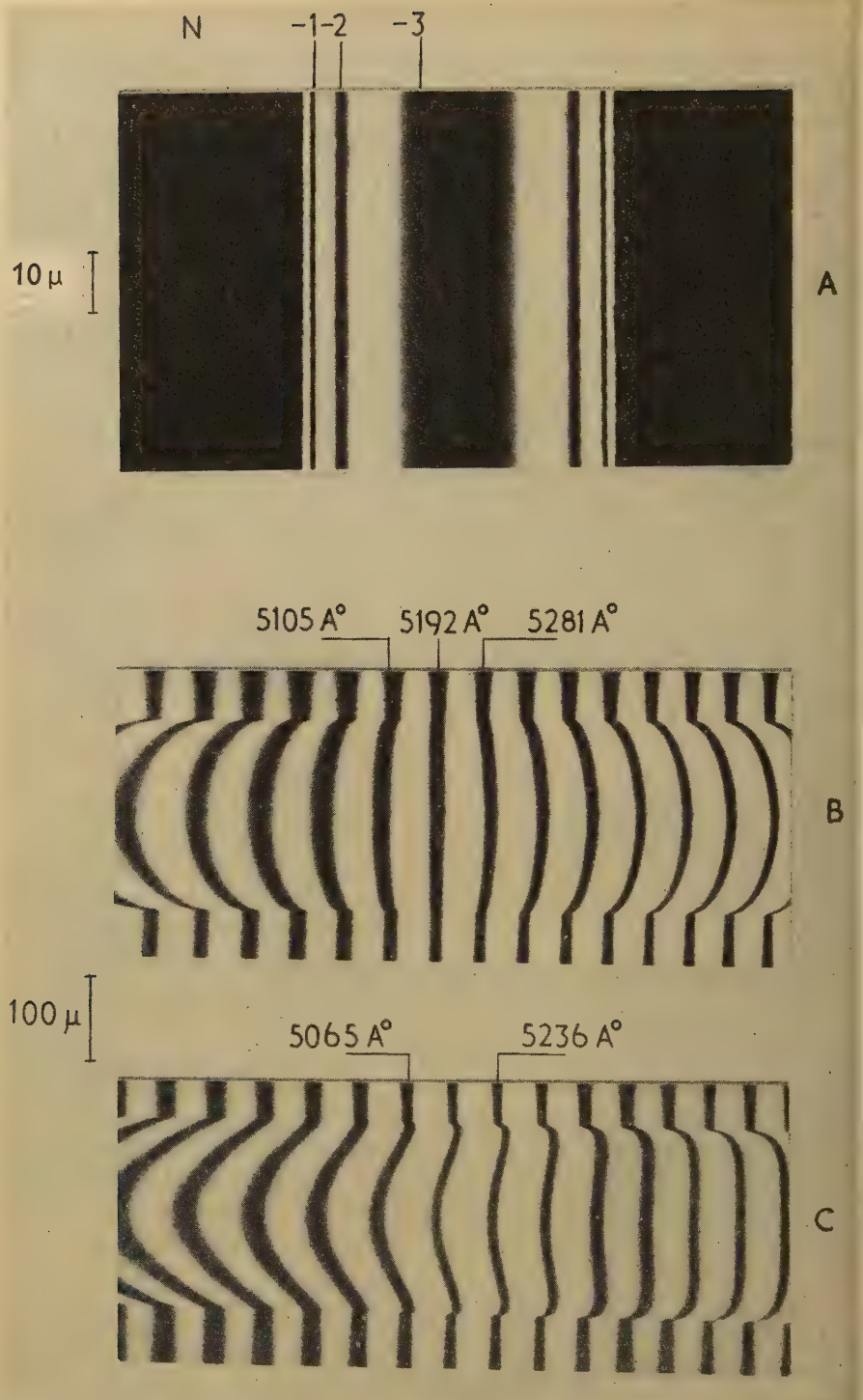

FIG. 3. A, intensity comparison. The ordinary image of an isotropic glass fibre. B and C, channelled spectra with a birefringent fibre. B and C were obtained from the ordinary images in the Y and X settings.

directions either parallel or perpendicular to that of the polarizer. It is then possible to insert in the eyepiece slot a quartz wedge with its line of greatest slope along the fibre axis (fig. 2, A and B). The resultant mutual inclination of the wavefronts W_1 and W_2 gives rise to a fringe pattern in monochromatic light. In the background the fringes will be at right angles to the fibre axis, but, upon entering the fibre, a given fringe will undergo a displacement equivalent to N times the separation between adjacent fringes. These displacements satisfy the relationship $2N\pi = \phi$, where ϕ is defined by equations (2) and (3). Since the quartz is cut with its slow axis along the line of greatest slope the following convention must be adopted: if the displacement is towards the thicker part of the wedge, N is negative for the Y setting and positive for the X setting. The direction of increasing wedge thickness is readily discovered from a study of the colour effects in white light. (If the phase difference introduced by the condenser-objective system acts in the same sense as that introduced by the wedge, there will be no point along the wedge at which the phase difference between W_1 and W_2 will be zero and, in the absence of the fibre, no achromatic fringe will be seen.)

The fringe displacement at any point of the fibre is therefore a direct measure of the phase difference there, and an exact picture of the index across the specimen can be obtained from a study of these displacements at different temperatures or wavelengths in accordance with the ideas expressed on p. 574. The application of the wavelength variation method presents no particular difficulties: it is simply necessary to keep the wavelength band passed by the monochromator as narrow as possible and to minimize the effects of a finite band-width by using an immersion liquid with a dispersion not greatly in excess of that of the fibre.

Fig. 4, A, B, shows the mercury green fringe patterns given by an unstretched viscose rayon model filament of 0·221 mm diameter in the Y and X settings; the $\times 10$ shearing system was used. Since it was intended to make a careful study of this fibre by another method (p. 582), it was not considered necessary to take a series of photographs at different temperatures or wavelengths. Instead fig. 4, A, B, was used as a guide for further work. Fortunately this model filament was a right cylinder and it was therefore possible to calculate the thickness at any part of the fibre. Equations (2) and (3) were then applied and the following information gained.

(i) In the ordinary image (Y setting) the axial fringe displacement is $N = -0{\cdot}10$ and, hence, $(\mu_{\parallel} - \mu_L) = -2{\cdot}4 \times 10^{-4}$. Near the fibre edge there is evidence of a slight positive displacement, but it is difficult to give precise figures because the displacement changes from one edge of the fibre to the other and from one fringe to the next. A reasonable average figure is $N = 0{\cdot}02$ at a distance of 0·012 mm from the edge; the calculated value of $(\mu_{\parallel} - \mu_L)$ is then $1{\cdot}0 \times 10^{-4}$. Consequently the mean index $\mu_{\parallel}$ at the fibre edge exceeds that on the axis by about 3×10^{-4}.

(ii) It is clear from the extraordinary image (Y setting) that the variation in $\mu_{\perp}$ is much greater. On the axis $N = 0{\cdot}23$ and $(\mu_{\perp} - \mu_L) = -5{\cdot}5 \times 10^{-4}$. At a

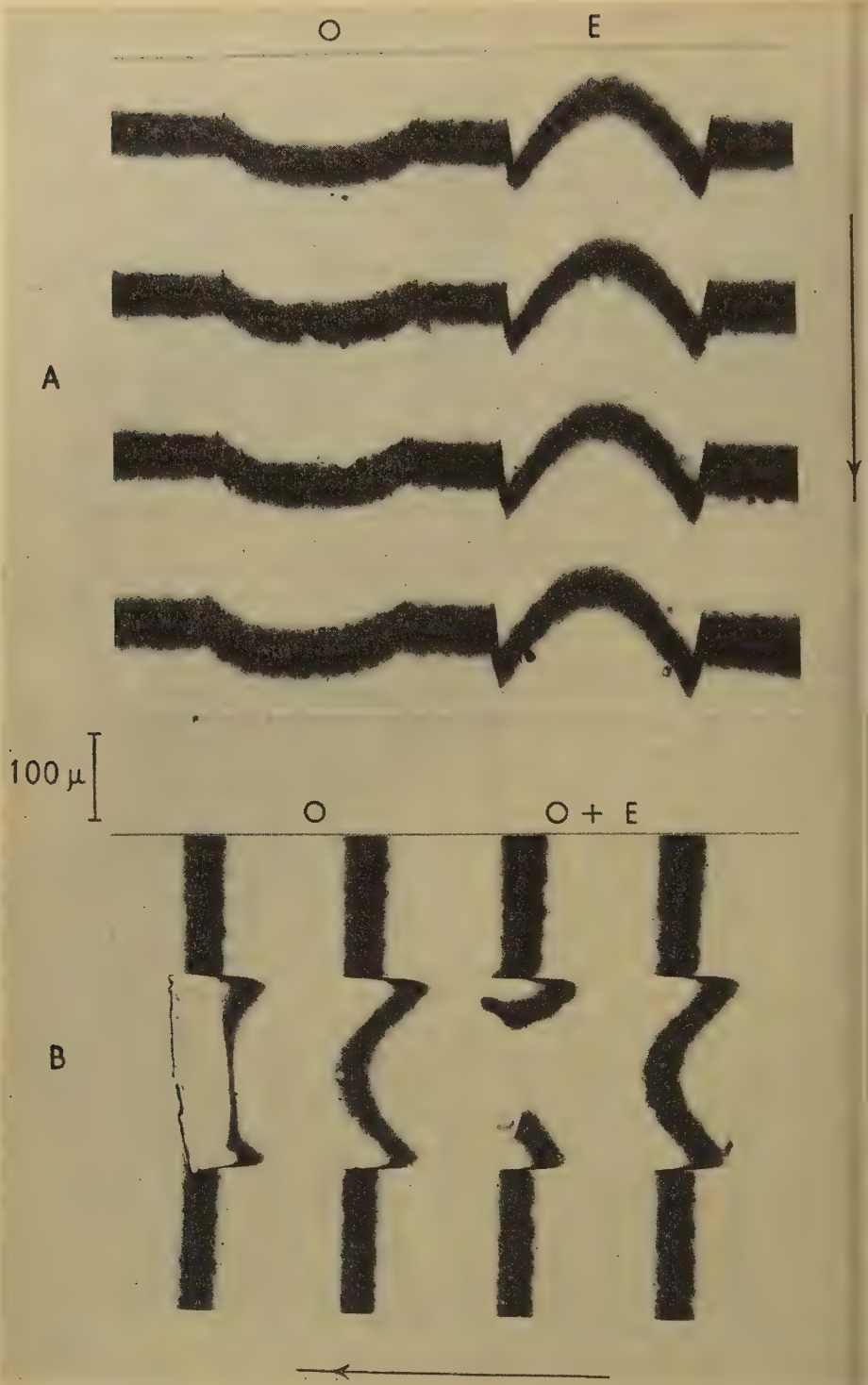

FIG. 4. Wedge fringes with a birefringent fibre in (A) the *Y* setting, and (B) the *X* setting. The arrows indicate the direction of increasing wedge thickness. *O* ordinary and *E* extraordinary image.

point 0·104 mm from the fibre edge $N = -0{\cdot}20$, giving a value of $9{\cdot}9 \times 10^{-4}$ for $(\mu_\perp - \mu_L)$. Hence the decrease in $\mu_\perp w$ from the edge to the axis of the fibre is at least 15×10^{-4}. The fringe displacement is zero at a point 0·045 mm from the edge.

(iii) The variation in $\mu_\perp$ is also revealed by the astigmatically-corrected ordinary image (X setting); this image is of short length and only one complete fringe can be seen. On the axis $N = -0{\cdot}04$ and $(\mu_\perp - \mu_L) = -1{\cdot}0 \times 10^{-4}$; at a distance of 0·012 mm from the edge $N = 0{\cdot}26$ and $(\mu_\perp - \mu_L) = 13{\cdot}6 \times 10^{-4}$. Here it is again found that the decrease in $\mu_\perp$ is 15×10^{-4} or more.

(iv) In fig. 4, A the axial value of $(\mu_\perp - \mu_L)$ is $-5{\cdot}5 \times 10^{-4}$ whilst in fig. 4, B it is only $-1{\cdot}0 \times 10^{-4}$. Since the temperature coefficient of refractive index for the liquid is about -4×10^{-4} per Centigrade degree and that for the fibre about -4×10^{-5} per Centigrade degree, the temperature must have increased by approximately one degree between the two exposures. This explains why the position of zero displacement is nearer to the fibre axis in fig. 4, B than in fig. 4, A.

(v) The overlapping images (X setting) show that the birefringence $(\mu_\parallel - \mu_\perp)$ is negative near the fibre edge but positive near the axis, a conclusion in conformity with the measurements on the individual indices $\mu_\parallel$ and $\mu_\perp$.

Near the fibre edge the vibration directions were not quite parallel and perpendicular to the axis. Although it was realized that this would cause a slight error (p. 588), the fibre was nevertheless selected because it so strikingly illustrated the difference between a fringe pattern obtained with a low index variation and that obtained with a high index variation.

The use of the achromatic fringe

Although the displacement N can usually be determined with the aid of a quartz wedge and monochromatic light, circumstances do occasionally arise in which it is impossible to decide which fringe in the specimen is connected to a given fringe outside; such conditions occur for a specimen with an abrupt edge, the fringes then being discontinuous. When this happens it is customary to use white light on the assumption that the achromatic fringe within the specimen is connected to the achromatic one outside. Unfortunately this assumption is valid only when the dispersion behaviour is the same for the various elements in the optical system. It can be shown (Faust and Marrinan, 1955) that if the true fringe displacement in mercury green light is N, the displacement N_A of the achromatic position (not achromatic fringe) is given by

$$N - N_A = -5{\cdot}85 \times 10^4 t(D_S - D_L), \tag{11}$$

where D_S and D_L denote the dispersions of the solid and the liquid between the F and C lines. In this equation a term containing the dispersion properties of the quartz wedge has been neglected, but the percentage error so introduced is only

$$\epsilon = 3(\mu_S - \mu_L)/(D_S - D_L). \tag{12}$$

For example, in the above experiment the dispersions of the rayon fibre and

the immersion liquid were 0·0092 and 0·0154 respectively, and $(\mu_S - \mu_L)$ for the mercury green light did not exceed 0·002. The error ϵ would therefore amount to one per cent. or less. Equation (11) then shows that on the fibre axis $(N - N_A) = 8{\cdot}02$. Thus, even if the axial displacement was zero at 5,461 Å, the achromatic position would be at −8·02 orders; that is, an achromatic fringe would be seen at −8 orders.

When such difficulties are encountered, two procedures are possible.

(*a*) If t and $(D_S - D_L)$ are known with sufficient accuracy, N can be calculated from equation (11). It must be noted that N_A, as judged by the position of the achromatic fringe or fringes, might be in error by as much as a quarter of an order. The errors due to other uncertainties must therefore be small if the calculated value of N is to be accurate to less than half an order. The true value of N will then be apparent from an examination of the fringe pattern in mercury green light.

(*b*) A crude value for the refractive index of the solid will already have been found from the Becke line method; this value will be in error by $d\mu_S$. If the solid is then immersed in a liquid of this same refractive index, $(\mu_S - \mu_L)$ will be equal to $d\mu_S$ and N will therefore differ from zero by the amount $d\mu_S\, t/\lambda$. For example, even if $d\mu_S$ is as large as 0·002, N cannot exceed a third of an order provided t is less than 80μ.

Channelled Spectra

With the two methods so far described it is necessary to examine a series of images or photographs, one at each temperature or wavelength, in order to obtain a complete picture of the variation of the mean index across the specimen. In the particular wavelength variation method now to be described only a single photograph is usually needed.

The microscope is set as explained on p. 577 with the exception that the quartz wedge is replaced by an accurately worked quartz plate of about 50 orders retardation, the vibration directions of this plate being X and Y. A white light source is used, the image of the fibre being so projected on to the slit of the spectrograph that the fibre axis lies at right angles to the slit length. The spectrum, instead of being continuous, is broken by a series of dark fringes, the effect being aptly described by the term 'channelled spectrum'. If the polarizer and analyser are crossed, these dark fringes occur at those wavelengths for which the path difference S between the two beams W_1 and W_2 is an integral number of wavelengths; that is, whenever

outside the fibre $\quad S^* = n\lambda_n^* = l\,\Delta\mu_n^* + p_n^*, \qquad (13a)$

inside the fibre $\quad S = n\lambda_n = l\,\Delta\mu_n + p_n + \lambda_n(\phi_n/2\pi), \qquad (13b)$

n being integral. If the polarizer and analyser are parallel, the same equations apply but n now has half-integral values. Here l is the thickness of the quartz plate and $\Delta\mu$ is its birefringence, which must be regarded as positive since the slow axis is always set along Y. The path difference p introduced by the condenser-objective system is the same for all parts of the image field; the setting

of the condenser screws should be such that p is as small as possible. The suffix n signifies that the quantities involved are referred to the fringe of order n.

It may be shown that

$$n(\lambda_n - \lambda_n^*)(1+B) = \lambda_n(\phi_n/2\pi), \tag{14}$$

where B is a small wavelength dependent term (see Faust, 1952). Since $l\,\Delta\mu$ is positive and greater than all the other terms, n is of necessity positive. For the ordinary images in either the X or Y setting ϕ is positive provided that the index of the solid exceeds that of the liquid. Under these circumstances a fringe of given order n will be of longer wavelength within the fibre than without. This behaviour, which is analogous to that of the corresponding multiple-beam fringes, is illustrated by fig. 3, B, C, which shows the ordinary images in the Y and X settings respectively; the specimen is a piece of the same fibre that was used for fig. 4, A and B. Towards the red the index of the solid exceeds that of the liquid and $\lambda_n > \lambda_n^*$; in the blue the reverse is true. For the Y setting there is at the wavelength 5,912 Å an almost perfectly straight fringe, from which fact it can be concluded that there is hardly any variation in $\mu_\parallel$ across the fibre width. For the X setting, however, there are several fringes for which there is zero displacement at some point across the fibre, and it must therefore be concluded that $\mu_\perp$ is far from constant.

In order to obtain a more complete picture of these index variations it is essential to know the difference between the dispersions of the liquid and the solid. Since the fibre is of circular cross-section, the thickness on the fibre axis is known and equation (9) can be used in order to obtain this dispersion difference. This equation involves a knowledge of ϕ at a number of different wavelengths, but equation (14) does not offer a convenient and accurate solution to this problem. A far better approach is to plot $n\lambda_n$ and $n\lambda_n^*$ against λ_n and λ_n^* respectively, the difference between these two curves giving the value of $(\mu_S - \mu_L)t$ at any desired wavelength; λ_n is measured on the fibre axis. Fig. 5 shows the curves obtained from the fringes in the X setting; before they could be plotted it was necessary to discover the order n, and for this purpose two procedures are theoretically possible.

The first is to employ the very close approximation

$$n = \frac{m\lambda_{n+m}^* + p_n^* - p_{n+m}^*}{(\lambda_n^* - \lambda_{n+m}^*)} \left[1 + k\frac{(\Delta\mu_F - \Delta\mu_C)}{\Delta\mu_n}\frac{(\lambda_n^* + \lambda_{n+m}^*)}{\lambda_n^*\,\lambda_{n+m}^{*2}}\right]^{-1} \tag{15}$$

relating the order n to the wavelengths of the fringes of order n and $n+m$. Here k is a constant whose value is $5{\cdot}23 \times 10^7$ when the wavelengths are expressed in Å. It is usually possible to ignore $(p_n^* - p_{n+m}^*)$ in comparison with $m\lambda_{n+m}^*$ and, if the dispersion curve of birefringence of the quartz is known with sufficient accuracy, the term in k can be calculated. The remaining error will be in the determination of the wavelength difference $(\lambda_n^* - \lambda_{n+m}^*)$, which has a value of approximately $100m$ (Å) in the middle of the spectrum. Since n itself is about 50 its value must be calculated to within $\pm\frac{1}{2}\%$ in order to

ensure that the correct integral value is taken for n. The wavelength of each fringe must therefore be measured to better than $\pm\frac{1}{4}m$(Å) and, as very accurate wavelength measurements cannot be expected with two-beam interference fringes, m should exceed 4.

The second and more satisfactory procedure was adopted. Since the path difference p is small and not markedly sensitive to wavelength, τ, the relative dispersion of birefringence of quartz, satisfies the approximate relationship

$$\tau = (\Delta\mu_F - \Delta\mu_C)/\Delta\mu_D \simeq (S^*_F - S^*_C)/S^*_D. \qquad (16)$$

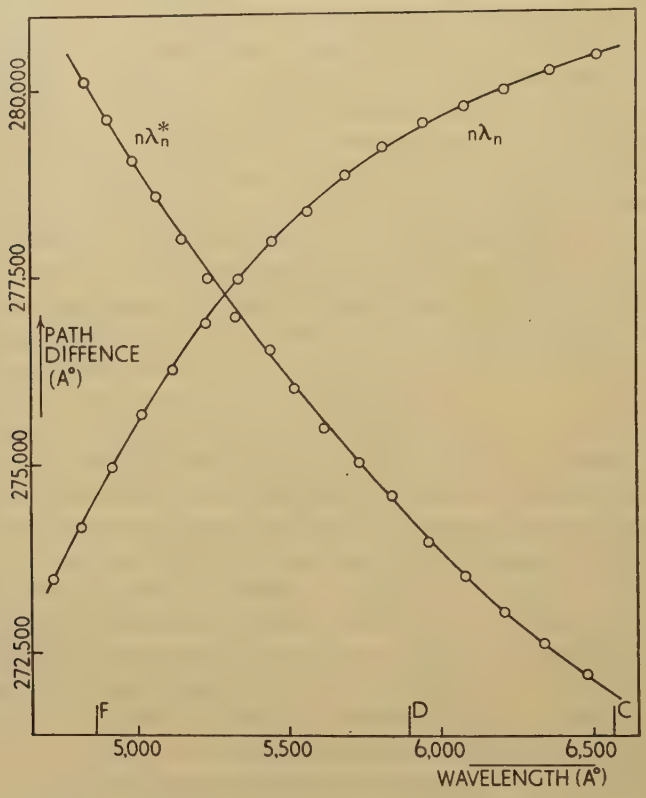

FIG. 5. The path differences S and S^* as functions of the wavelength.

The order n is therefore so chosen that the value of τ calculated from equation (16) agrees closely with the value of 0·0292 obtained from the International Critical Tables. For example, in fig. 5 the selected order for the fringe at the wavelength $\lambda^*_n = 5{,}236$ Å was 53, leading to a τ value of 0·0289. The orders 52 and 54 were clearly wrong because the calculated values of τ would then be 0·0357 and 0·0221 respectively.

Once the correct order had been ascertained, it was found from fig. 5 that the dispersion difference $(D_\perp - D_L)$ was equal to $-0{\cdot}0062 \pm 0{\cdot}0001$. Since the dispersion D_L of the immersion liquid was $0{\cdot}0154 \pm 0{\cdot}0001$, the dispersion $D_\perp$ of the fibre for light vibrations at right angles to the fibre axis was

0·0092±0·0002. Because the birefringence of the fibre was extremely small, it was assumed that $D_\perp$ and $D_\parallel$ were equal. (This assumption is not generally valid; e.g. a cellulose fibre with a birefringence of 0·0238 has a dispersion difference ($D_\parallel - D_\perp$) of about 0·0004; see Faust and Marrinan, 1955.)

With the aid of this information it was then possible to discover the way in which the mean indices changed from one region of the fibre to another: $\mu_\parallel$ was constant to within ±0·0001, but $\mu_\perp$ was greater at the edges than at the

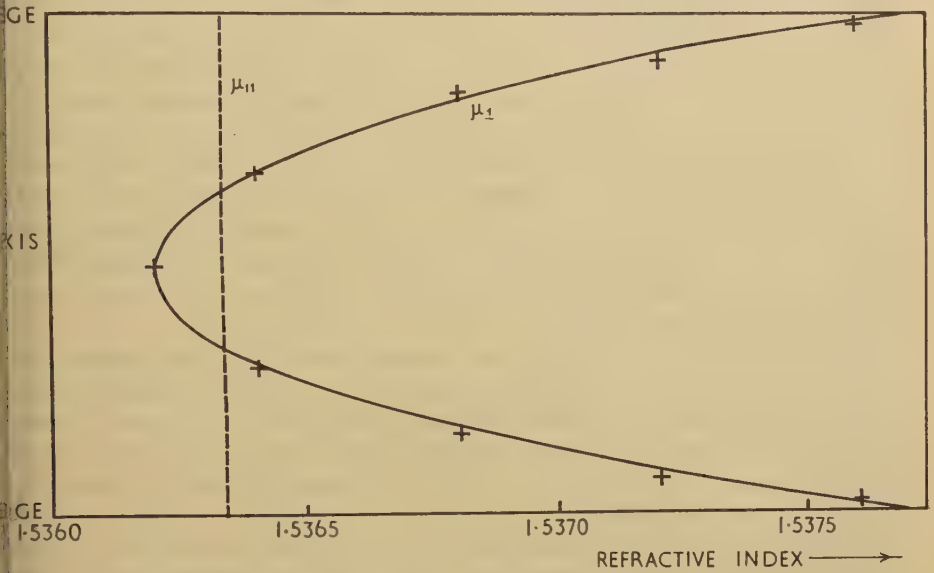

FIG. 6. The variation of the mean indices $\mu_\parallel$ and $\mu_\perp$ from one edge of the fibre to another.

axis by 0·0015 (fig. 6). These figures are consistent with those quoted in the previous section for a similar piece of fibre.

With the quartz plate used in these experiments the wavelength separation between the fringes in the background is about 100 Å in the middle of the visible spectrum. Since ($D_S - D_L$) is 0·0062, a wavelength change of 100 Å corresponds to a change of 0·0004 in ($\mu_S - \mu_L$). Consequently the index of the fibre at a point of zero displacement on a given fringe differs by 0·0004 from the index of the fibre at the point of zero displacement on the next fringe (compare fig. 6). If it is desired to obtain closer index readings two photographs can be taken, one with the polarizer and analyser crossed, the other with them parallel; in this way the index readings are only 0·0002 apart. Even closer readings can be obtained by interpolation between fringes; for example, in fig. 3, c there is no fringe for which the fringe displacement is zero on the fibre axis, but the intersection of the two curves in fig. 5 yields the wavelength at which the indices of the solid and liquid are equal. Furthermore, it would have been possible to obtain even closer index readings by the use of an immersion liquid with a smaller wavelength dispersion.

Accuracy

In order to study the index variations within a specimen it is necessary to discover, at different temperatures or wavelengths, those points of the specimen for which the phase difference is zero, and then to calculate the index at those points from equation (5) or (8). This procedure involves three distinct sources of error.

(i) The index $(\mu_L)_{\theta_0}$ or $(\mu_L)_{\lambda_0}$ of the liquid will be in error by $\pm 0{\cdot}0001$ or less. Since this uncertainty is common to all the measurements it will not affect refractive index differences within the specimen.

(ii) The experimentally determined 'points of zero phase difference' will be points at which the phase difference is close to but not necessarily equal to zero. Under favourable circumstances this phase difference should not exceed $\pm 1°$ with the intensity comparison technique, $\pm 7°$ with the wedge fringes and $\pm 3°$ with the channelled spectra. A phase uncertainty of $\pm 1°$ will cause the calculated values of $(\mu_S)_{\theta_0}$ or $(\mu_S)_{\lambda_0}$ to be in error by $\pm\lambda/360t$, which amounts to $\pm 0{\cdot}00016$ for a specimen thickness of 10 μ. The intensity comparison is therefore the best technique for the examination of small index variations in very thin specimens.

(iii) The determination of the difference between the temperature coefficients or the wavelength dispersions of the liquid and the solid can be made in one of two ways: (*a*) the properties of the liquid and the solid can be measured separately, or (*b*) the difference in properties can be calculated from the phase measurements made at a point of known thickness on either the specimen or standard object.

Procedure (*a*). Since the specimen is heterogeneous, the same region must be examined in each immersion liquid, and the ease with which this can be done will depend upon the nature of the specimen. However, under good conditions, the error in the difference between the temperature coefficients should not exceed $\pm 0{\cdot}00002$ per Centigrade degree for a specimen 10 μ thick. If the index variation within the specimen is 0·0020, a temperature change of about 5° will be needed and the associated error will be approximately $\pm 0{\cdot}0001$. Similarly, the difference $(D_S - D_L)$ between the wavelength dispersions should be in error by less than $\pm 0{\cdot}0004$ for a specimen 10 μ thick, and the error involved in the measurement of an index variation of 0·0020 will not exceed $\pm 8\times 10^{-7}/(D_S - D_L)$; if $(D_S - D_L)$ is $-0{\cdot}0060$, this maximum error is $\pm 0{\cdot}00015$.

Procedure (*b*). Here errors arise from uncertainties in the phases ϕ'_{θ_0} and ϕ'_{θ} and in the thickness t' of the specimen or the standard object at the point Q (equations (6) and (9)). If each phase measurement is subject to an error of $\pm 1°$, the associated index error can be as great as $\pm\lambda/180t'$, which amounts to $\pm 0{\cdot}0003$ for a thickness of 10 μ. The other error is equal to dt'/t' times the difference, at θ_0, between the index of the solid at P and that at P_0. Hence, if this index difference is 0·0020, a 5% error in the thickness will yield an index error of $\pm 0{\cdot}0001$. (θ_0 and θ could equally well be λ_0 and λ.) This procedure will therefore give accurate results only when t' exceeds 30 μ. The first error

will then be $\pm 0{\cdot}0001$, and the second one should be no greater. Satisfactory thickness determinations can be made by the method of apparent depth (Brattgård, 1954; Galbraith, 1955), by rotation of the specimen through 90° about an axis at right angles to the microscope axis, by interferometry (Denton, 1952), or by the use of two immersion liquids as described in the Introduction.

Two-beam and Multiple-beam Interference

This section outlines the main differences between the Baker two-beam interference techniques and the corresponding multiple-beam ones.

(i) The evaporated metallic films used with multiple-beam interference result in light losses that make it difficult to work at high magnifications. Each metallic film could possibly be replaced by several dielectric layers, but, as a freshly coated optical flat is required for each immersion liquid, this is not an attractive solution to the problem.

(ii) Multiple-beam fringes are often extremely sharp; furthermore, their displacement is proportional to 2ϕ. Thus, under good conditions, the multiple-beam system is much more sensitive than the two-beam one. However, since the light passes through the specimen many times, the phase relationships within the object are not so accurately portrayed as with the two-beam system. The magnitude of this phase distortion will depend upon the fineness of the structure within the object and upon the thickness of the object; with very thin objects it is insignificant (Faust, 1952).

(iii) Birefringent specimens in which the vibration directions are not constant can readily be examined under a multiple-beam interference microscope.

(iv) With multiple-beam interference the lateral dimensions of the specimen are unimportant, and sheet specimens can therefore be studied.

(v) In the Baker microscope the background phase ϕ_B is controlled by the instrumental arrangement (e.g. the quartz wedge or plate), and it is possible to have low orders of interference with thick specimens. With multiple-beam interference the order of interference is determined by the closeness of approach of the two optical flats; i.e. by the maximum specimen thickness. For instance, a specimen 0·01 mm thick leads to an order of about 50.

(vi) If the optical flats are parallel and illuminated with monochromatic light, the phase differences within the specimen will be revealed as intensity changes in the multiple-beam interference pattern. This method, which is analogous to that described on p. 576, is extremely sensitive at very low orders of interference, and points of zero phase difference can therefore be accurately found. If, however, it is desired to measure a phase difference other than zero, photometric devices must be used in order to make the necessary intensity measurements.

(vii) The multiple-beam equivalent of the quartz wedge technique is produced by inclining the optical flats at a small angle to one another. The major difference is that with multiple-beam interference the order of interference is usually so high that the incident radiation must be of narrow line width, the

most satisfactory spectral line being the mercury green one; the inability t use a monochromator prevents one from using the wavelength variatio method.

(viii) The multiple-beam equivalent of the quartz plate technique involve the use of parallel optical flats illuminated with white light, the resultant inter ference pattern being analysed with the aid of a spectrograph. In the expres sion corresponding to equation (13) $\Delta\mu$ is replaced by the index μ_L of th liquid, l by the separation t_g of the interferometer plates, and p by a term Ω that represents, as a fraction of the wavelength, the reflection phase change suffered at the metallic films (Faust, 1952). Unlike p this term is highl sensitive to wavelength, its value at the C line exceeding that at the F line b about 1,700 Å. (For the silver films normally used in interferometry $\Omega_C \simeq 0$ and $\Omega_F \simeq 0{\cdot}6$ (Faust, 1950).) Thus, if n is to be calculated from the equivalen of equation (15), the wavelength dependence of Ω must be accurately known Furthermore, n cannot be calculated from the expression corresponding t equation (16), because the approximation is too coarse and a correction term based on the wavelength behaviour of Ω must be introduced. Because of these difficulties it will not always be practicable to determine the order n unam biguously. Although in such instances it will still be possible to determine th positions of zero fringe displacements (i.e. zero phase differences), it will no be possible to measure accurately a phase difference ϕ other than zero. Thi means that the difference between the dispersions of the liquid and the solid cannot then be determined from the multiple-beam fringe pattern even though the specimen thickness is known.

Incorrect Orientation of the Specimen

Fig. 1 (p. 571) is based upon the assumption that the condenser aperture i so small that the diameter of the Airy disk is greater than the distance o shear. Each point of the light source would then produce two coherent dis turbances, one with vibrations along X and one with vibrations along Y, a any given point of the object plane. In practice, however, the Airy disk i much smaller than the shear distance, and each source point produces only one disturbance at a given object point. Thus, a particular point P of the specimen will be illuminated by two incoherent disturbances, one originating at the source point L and one at the source point L'. Their complex amplitudes will be denoted by Y_c and X'_c respectively, the suffix c signifying that these disturbances have passed through the condenser and associated half-wave plate. The disturbance X_c, arising from L and coherent with Y_c, is displaced through the shearing distance.

If a birefringent fibre with vibration directions parallel and perpendicular to the fibre axis is set with its axis at an angle α to Y, the vibration direction of the light emerging from the fibre are no longer along X and Y. However when the light encounters the objective, the vibration directions are restored to X and Y. If the complex transmission factors of the objective are denote

y X_0 and Y_0, the disturbance issuing from the objective with its vibrations long Y consists of two incoherent parts:

$$U+U' = RY_0\, Y_c \exp i(\phi_{\|}+\chi)+R'Y_0\, X'_c \exp i(\phi_{\|}+\chi'), \tag{17}$$

where

$$R = (1-\sin^2 2\alpha \sin^2 \tfrac{1}{2}\Delta)^{\frac{1}{2}}, \tag{18a}$$

$$R' = \sin 2\alpha \sin \tfrac{1}{2}\Delta = (1-R^2)^{\frac{1}{2}}, \tag{18b}$$

$$\tan \chi = \frac{\sin^2\alpha \sin \Delta}{\cos^2\alpha+\sin^2\alpha \cos \Delta}, \tag{18c}$$

$$\tan \chi' = -\frac{\sin \Delta}{1-\cos \Delta}. \tag{18d}$$

Here Δ represents the birefringent phase difference $(\phi_{\perp}-\phi_{\|})$.

The objective also removes the shear introduced by the condenser with the result that the disturbance $X_0 X_c$ is superimposed upon the disturbance $(U+U')$. Coherence considerations show that when an analyser is introduced so as to bring these disturbances into a common vibration direction, interference is possible only between $X_0 X_c$ and U. Hence, whatever method of phase measurement is used, an examination of the ordinary image suggests that the fibre introduces a phase difference $\phi = (\phi_{\|}+\chi)$. A few values of χ are included in the accompanying table: the positions $\alpha = 0$ and $\alpha = \frac{1}{2}\pi$ corre-

α	χ	$(\phi_{\|}+\chi)$	R
0	0	$\phi_{\|}$	1
$\frac{1}{4}\pi$	$\frac{1}{2}\Delta$	$\frac{1}{2}(\phi_{\perp}+\phi_{\|})$	$\cos \frac{1}{2}\Delta$
$\frac{1}{2}\pi$	Δ	$\phi_{\perp}$	1
α small	$\alpha^2 \sin \Delta$	$(\phi_{\|}+\alpha^2 \sin \Delta)$	$(1-2\alpha^2\sin^2 \frac{1}{2}\Delta)$

pond to the correct alignment of the fibre for the Y and X settings of fig. 2 p. 573). When α is small, it is usually safe to neglect χ, since its maximum value is only $\pm\alpha^2$.

However, even when α is small, the accuracy of the phase measurements will be reduced because of the presence of the disturbance U'. If a quartz wedge or plate is used and the analyser is set at 45° to X and Y, the resultant image intensity will satisfy the proportionality relationship:

$$I \propto |U+X_0 X_c|^2+|U'|^2. \tag{19}$$

For a correctly aligned microscope it can be assumed that $|X'_c|=|X_c|=|Y_c|$ and that $|X_0| = |Y_0|$, whereupon the relationship becomes

$$I \propto [1+R \cos (\phi_B+\phi_{\|}+\chi)], \tag{20}$$

where ϕ_B is the total phase difference introduced by the condenser-objective system and the quartz wedge or plate. If the quarter-wave plate method is used the cosine term is replaced by $\sin (\phi_B+\phi_{\|}+\chi+2\gamma)$, where γ is the angle between the analyser direction and the X axis, and ϕ_B is simply the

phase difference introduced by the condenser-objective system. The ratio of minimum to maximum intensity is thus $(I-R)/(I+R)$ which, for a given value of Δ, approaches unity most closely when $\alpha = \frac{1}{4}\pi$.

Wavelength Sensitivity of the Half-wave Plate

For reasons of manufacture an achromatic half-wave plate *HH* is used only in the ×10 and ×40 double-focus systems. If *HH* is not strictly a half-wave plate, phase measurements will not be subject to any systematic error, but their accuracy will probably be reduced as a result of a decrease in the contrast of the interference pattern. For instance, if *HH* introduces a phase difference of $(\pi+\beta)$, both Y_c and X_c will consist of two incoherent terms; that is $Y_c = y+y'$ and $X_c = x+x''$, where x and y are coherent but x, x'', and y' are not. It is readily shown that $|x| = |y|$ and $|x''| = |y'|$, and also that

$$|x''| = |x| \tan^2 \tfrac{1}{2}\beta.$$

Hence, when X_c and Y_c have traversed the specimen, the objective, and the analyser, the ratio of minimum to maximum intensity is

$$\frac{I_{\min}}{I_{\max}} = \frac{|x-y|^2+|x''|^2+|y'|^2}{|x+y|^2+|x''|^2+|y'|^2} \qquad (21a)$$

$$= \frac{\tan^2 \tfrac{1}{2}\beta}{2+\tan^2 \tfrac{1}{2}\beta}. \qquad (21b)$$

Thus, as expected, the contrast will be greatest when $\beta = 0$, that is whenever *HH* is a true half-wave plate, and will completely disappear when $\beta = \pm\pi$, that is whenever *HH* is a full-wave plate.

If β is small, the above ratio can be expressed as $\beta^2/8$. For example, *HH* is designed for use with 5,461 Å radiation and, if the incident light has a wavelength of 5,000 Å, β is equal to 0·28 radians. The ratio of minimum to maximum intensity will then be 0·01. Although this ratio is probably significant in the intensity comparison method, it is not sufficiently large to disturb the definition of the fringes shown in fig. 3, B and C.

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A Modified Ester Wax for Embedding Tissues

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Summary

1. A modification of Steedman's ester wax embedding method is described. This involved purification of two of the components—diethylene glycol monostearate and distearate. The puckering and floating off of the sections is thereby minimized.

2. The method is particularly applicable to tissues containing much collagen and to tissues that have been badly fixed or which have undergone post-mortem degeneration.

3. Cytological preservation is improved, especially that of such substances as glycoproteins and mucoproteins.

ORTON and Post (1932) and Cutler (1935) investigated the use of diethylene glycol distearate as an embedding medium. The unsatisfactory results were partially overcome by Steedman (1947), who added other components in varying proportions. He examined many different mixtures and investigated with great care the distortion of the tissues produced during embedding, cutting, and mounting. The final mixture chosen has been called ester wax.

Although ester wax has been strongly recommended (Gatenby, 1950), it does not seem to have been realized that, quite apart from diminution of gross shrinkage of the specimen, the preservation of cellular detail is much improved. Were it not for the difficulty of handling the blocks and sections, undoubtedly ester wax would have come into wider use. Those who have tried using it appear usually to have abandoned it because of the following technical difficulties:

1. The blocks contract excessively after casting and are apt to crystallize in the centre. They are brittle and difficult to trim.

2. The sections when mounted on the slide are usually puckered and are apt to become detached during staining.

We have attempted to make the method technically easier to use, without losing the good preservation of the tissues that can be obtained by use of ester wax. It was found that one of the ingredients, diethylene glycol monostearate, was impure and that the amount of impurity was very variable. A modification of ester wax, based on the use of a pure sample of the monostearate, was worked out. By arrangement this modification has already been published elsewhere by Heatley and others (1956).

The modification described here gives further improvement in ease of cutting and handling, and slightly better preservation of the tissues. This is obtained by using a pure sample of the diethylene glycol distearate.

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The wax mixture

The best proportions are found to be:

diethylene glycol monostearate (purified) 100 g.
diethylene glycol distearate (purified) 100 g.
castor oil 5 g.

This mixture may be obtained suitably blended, ready mixed, and filtered from the Watford Chemical Co. Ltd., 22 Copperfield Road, Canal Road, London, E. 3. It is sold under the trade name of 'Estax Brand Wax, Histological Grade'.

Embedding technique

Melt a suitable amount in the oven at 53° C the day before it is required. It must not be melted by rapid heating over a Bunsen burner. It must not be stored for days in the oven because it slowly decomposes at high temperatures. It is also slightly unstable when dissolved in benzene. The melting-point of this wax mixture is about 48° C.

Although ester wax and our modification of it may be used after any of the usual dehydration and clearing procedures, we strongly advise careful and complete dehydration, subsequent removal of all traces of alcohol and clearing fluid, and prolonged infiltration with the wax. This neither hardens the tissues unduly nor causes excessive shrinkage; on the contrary, sections are easier to cut and structure is better preserved both with paraffin and with ester wax embedding. Langeron (1925) gives similar advice and quotes P. Masson's apt phrase, 'L'aspect cuit tient à un défaut de cuisson'. The following times are those suitable for blocks that are about 5 mm thick:

1. Fix by any desired fixative; wash out the fixative as usual.
2. 70% alcohol, 12 hours.
3. 90% alcohol, 12 hours.
4. 96% alcohol, 12 hours.
5. Absolute alcohol, 12 hours; change once.
6. Absolute alcohol, 12 hours.
7. Absolute alcohol and xylene (equal volumes); change twice, overnight.
8. Xylene—2 hours or until cleared.
9. Modified ester wax in an oven at 53° C—overnight or up to 24 hours. Change once or twice after an hour or two. Do not employ wax that has already been used.
10. Cast in fresh wax in L-moulds on a flat metal plate; fill up whilst cooling.

The retraction during cooling is no greater than with paraffin wax; crystallization is rare. The blocks can be trimmed easily, especially if a warmed knife or 'junior' hacksaw is used.

Steedman (1947) advised the use of cellosolve to replace alcohol and xylene prior to infiltration. We find that this further improves the preservation of

structure. The technique is simple: fix, wash if necessary; transfer to cellosolve changing at least 4 times during 24 hours; transfer direct to modified ester wax.

Section cutting and mounting

Sections should cut at least as easily and at least as thin as similar material embedded in paraffin wax. The optimum thickness is usually 6 μ. Material containing much fibrous tissue such as skin, uterus, and urinary bladder cut more easily than when embedded in paraffin. Room temperature for sectioning should not exceed 65° F. The sections ribbon very easily. They show fine puckerings, but these disappear during flattening on to the slide.

Sections are cut off the ribbon and put on the surface of a bowl of cold water containing a synthetic detergent (for instance, 4 drops of 'teepol' in 200 ml of distilled water). They are taken on to a slide, drained of excess fluid, and put on a hot plate to flatten. The temperature required is lower than that for paraffin sections. It can be attained by using a 31° C capsule and adjusting the control until a piece of wax nearly melts on top of the plate. Alternatively put a sheet of plate glass $\frac{1}{4}$ in. thick on top of a plate adjusted for paraffin sections. The crinkles disappear from the sections; if the temperature is not quite high enough put a drop of dilute teepol solution between the slide and the plate. Drain off excess fluid and allow the slide to dry at a lower temperature on a hot plate. Complete the drying in an oven at 37° C overnight. Drying is quicker than with paraffin sections; one hour's drying is quite adequate if speed is essential.

Sections adhere well to the slide but not quite as well as corresponding paraffin sections. If complicated staining procedures are to be used, the slides should be coated in celloidin in the usual way.

Results

Detachment or folding of the sections may be slightly more frequent than with paraffin sections, but with practice this fault becomes negligible.

The following are the main improvements over the use of paraffin wax that have been found so far:

1. The general preservation of all tissues is improved; shrinkage is diminished.
2. The preservation of cytological detail is better.
3. Bad fixation yields less distortion.
4. The preservation of glycoproteins and mucoproteins is better. After Helly fixation many kinds of epithelial mucin are retained in granular form. This has been confirmed by Heatley and others (1956).
5. Post-mortem degeneration is less obvious.
6. Fibrous material cuts better and there is less shrinkage of collagen fibres.
7. Sections of the nervous system are less liable to show 'cracking'; small blood-vessels do not detach and leave empty spaces, as they sometimes do from paraffin sections.

8. It is unnecessary to use vacuum embedding for lung tissue. The preservation, even of post-mortem material, is good.

Staining results are somewhat affected. The sections have a greater affinity for basic dyes and dye-lakes such as those of haematoxylin; 2 to 3 minutes are ample for most types of haematoxylin stain. Methods such as Masson's trichrome may need slight modification of staining times.

Discussion

It was realized by Hardie and Wesbrook (1895) that damage could result from exposure of paraffin sections to water. It seems that the paraffin crystals are not small enough or closely enough packed to protect the dried proteins in the section from the swelling action of water. Indeed, the tissues in a thin paraffin section can often be seen to swell laterally during flattening on the slide. Again, if the cut surface of the tissues in a paraffin block are exposed to water, they swell and are squeezed out of the wax. Presumably as the section dries again on the slide, the tissues will tend to contract after adherence to the slide has begun. It is perhaps surprising that such violent mechanical distortions produce so little apparent damage.

For demonstration of glycogen in sections it is often advised that 70% alcohol should replace water for flattening sections. Leach (1938) suggested the use of 96% alcohol when mucoproteins are present. Carleton and Leach (1939) found much improvement in many techniques when diacetin was used; post-mortem degeneration was minimized.

The celloidin of the Peterfi double-embedding process seems to give to the tissues mechanical protection from the swelling action of water (Leach, 1947). The modified ester wax has a soapy, uniform, non-crystalline structure and penetrates the minutest interstices of the tissues; it is probably because of this nature that it acts so effectively in preventing the swelling and dissolving action of the water used for flattening the sections. Smyth and Hopkins (1948) offered a similar explanation to account for the excellent results obtained by embedding tissues in ester wax when glycogen is to be demonstrated. They offered no explanation for their observation that after removal of the wax and treatment with alcohol, exposure to water no longer caused damage.

It might be thought that the protection offered by ester wax to the swelling action of water was pointless when the sections were to be exposed to water during the staining process, but the explanation lies in the treatment of the sections with alcohol after the removal of the wax by xylene. It has been shown (Leach, 1945) that greatly improved preservation of tissues can be obtained by treating tissues, after clearing in xylene, with alcohol; the tissues are then re-cleared and embedded. It was suggested that removal of fat exposed proteins which could then be further denatured by alcohol. If this post-fixation in alcohol is not employed, then such undenatured proteins will be present in wax sections. Contact with water would cause solution or distortion. But if this is minimized by use of celloidin or ester wax, then the treatment of

the sections with alcohol before staining will ensure the post-fixation of the undenatured protein and so prevent the damage by the aqueous staining solutions.

The correctness of this assumption has been shown thus. A piece of the duodenum of the rabbit was fixed in Helly's fluid and embedded in ester wax. Sections were flattened on slides. One section was treated in the usual way and stained for mucin by the PAS method. Another section, after removal of the ester wax with xylene, was taken direct to running tap water without treatment with alcohol. After all the xylene had been removed the section was also stained by the PAS method. The mucin was not nearly so well preserved as in the section treated in the normal way. This shows that the treatment of the sections with alcohol, after removal of the fatty substances of tissues with xylene before and after embedding, causes further fixation of mucoproteins. If, however, a similar piece of tissue was postfixed in alcohol before embedding, the omission of alcohol in the treatment of the sections had no appreciable effect.

It is understandable that tissues that have been weakened by post-mortem degeneration or imperfectly fixed will show the most marked improvement when protected by ester wax from the action of water. This corresponds to the similar observations that have been made when the same end was achieved by flattening the sections on diacetin (Carleton and Leach, 1939) or by postfixation of the tissues (Leach, 1945).

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The Development of the Malpighian Tubules of *Schistocerca gregaria* (Orthoptera)

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Summary

1. *Schistocerca gregaria* Forsk. possesses about 250 Malpighian tubules arranged in twelve groups, situated dorso-laterally, laterally, ventro-laterally, and in the six intervening positions around the posterior end of the mid-gut. The gut forms an expanded ampulla at the point of entry of each group.

2. Each tubule is composed of five rows of cells, spirally arranged. Upper and lower segments are present, as in *Rhodnius* (Wigglesworth, 1931), the former very short. There is also a spiral trachea.

3. Tubules arise in seven generations, two in the embryo and one in each of the five nymphal instars. The first embryonal generation is composed of six primary tubules, the second of twelve embryonal secondaries. In the other five a variable number of nymphal secondaries is added, with means of 25, 51, 88, 63, and 6 respectively. The six primary tubules are attached distally to the rectum, the embryonal and first and second instar secondaries pass forwards, the fourth and fifth instar secondaries pass backwards, and the third in both directions.

4. The development of any one tubule comprises a period of initiation, a period of mitosis and elongation, and a period of differentiation. The first is restricted to the first few days of the particular stadium, the second takes place throughout the stadium, and the last throughout the life of the tubule.

5. The secondary tubules are arranged in an arc round the periphery of the ampulla. These arcs are perfectly symmetrical, the tubules arising alternately left and right of the mid-line during all the nymphal stages.

6. Tubule buds formed late in the period of initiation may cease to develop until the corresponding phase of the succeeding instar is reached.

7. The origin of tubules from a posterior interstitial ring and its relation to the interpretation of insect gastrulation and the nature of nymphal developmental cycles is discussed.

Introduction

ALTHOUGH the embryogenesis and metamorphosis of insects have been studied for many years, the intermediate phases of larval development have been comparatively neglected. One aspect of this wider field of development, that which concerns Malpighian tubules, has been investigated in only a few cases, i.e. *Blatta*, *Forficula*, and *Pieris* (Henson, 1944, 1946*b*, 1947). The results proved to be significant in relation to the general theory of insect gastrulation on the one hand, and to the relationships between embryogenesis and metamorphosis on the other. Furthermore, these results revealed that the cellular constitution of the tubules and their histogenesis are different in each of the forms investigated. An understanding of these differences seems likely to depend upon further knowledge of other types.

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Malpighian tubules have also been made an important element in the primary classification of insects (Martynov in Grassé, 1949), and so from this point of view it is important that their morphological relationships should be properly understood.

It is evident that further studies of the development of Malpighian tubules in a wider range of types could materially contribute to a better understanding of all these problems. The Orthopteroid groups present a sufficiently wide range of form to make them favourable material for such studies and a beginning is made in the present paper with *Schistocerca gregaria* Forsk. It is hoped to study other Orthopteroids later.

Materials and Methods

The locusts were bred in the laboratory through all stages from egg to adult. The eggs were kept at 33° C for about 2 days, to prevent the onset of diapause, and then at 25° C for a further period of about 17 days until the nymphs hatched. The latter were then reared in the usual type of breeding cage. The identification of the six instars was based on Uvarov (1928).

Examples of all stages were dissected and examined in either 1% saline or 30% alcohol or after overnight staining in methylene blue in 1% saline. For microscopic preparations material was fixed in Carnoy's fluid, double-embedded in celloidin and paraffin wax, and stained in Delafield's or Heidenhain's haematoxylin with eosin or orange G as counter-stains. Whole mounts were stained in Delafield's haematoxylin.

The range of variation in the number of tubules has not been statistically investigated since it is not significant for the problems here discussed. Nevertheless, some 200 specimens have actually been examined.

The Anatomy of the Tubules

The number of Malpighian tubules in an adult locust is roughly 250. They are not produced simultaneously but arise in successive series in all phases of the life-history except the imago. There are seven series in all, two generations in the embryo and one in each of the five nymphal instars. The first generation arises in the early embryo and consists of six tubules; the second generation consisting of twelve tubules arises in the late embryo: the third to the seventh generations arise in the first to the fifth nymphal instars and consist of a variable number of tubules with means of 25, 51, 88, 63, and 6 respectively. Thus 18 tubules are present at the time of hatching and a further 233 or so are added during nymphal stages. The tubules of each of these 7 generations may be named according to the time of their formation. The 6 first formed embryonal tubules are the 'primaries', the 12 later formed embryonal tubules are the 'embryonal secondaries', whilst the remainder are 'nymphal secondaries'. These latter are 'first instar secondaries', 'second instar secondaries', and so on.

The anatomy of the tubules thus varies appreciably according to the stage

nder consideration, and under the present heading attention is confined to ıe adult and the embryo.

Adult. The adult locust possesses 220–94 tubules arranged in 12 groups round ıe hinder end of the mid-gut and definitely orientated in relation to it, occupy- ıg dorso-lateral, lateral, ventro-lateral, and the intervening positions (as in g. 1, A). In each of these groups there are 21 to 28 tubules, all of which open ıto an anteriorly directed, flattened, digitate ampulla situated just anterior to ıe large sphincter muscle round the anterior end of the hind-gut. The latter robably corresponds to the ileo-ventricular muscle of *Melanoplus* (Stuart, 935), but its more posterior position, entirely on the hind-gut, renders exact orrespondence doubtful (figs. 1, D; 3). The ampullae consist of mid-gut ells anteriorly and hind-gut cells posteriorly, and each opens into the lumen f the gut by a transversely elongated pore. A deep annular groove connects hese pores round the circumference of the gut. The line of demarcation of nid-gut and hind-gut runs across the ampullae and along the bottom of the nnular groove. Behind each pore the hind-gut is thrown into a deep longi- udinal fold which provides an ample channel for the flow of 'urine' in spite of he fact that the peritrophic membrane extends back into the hind-gut over he openings of the ampullae (fig. 3). This backward extension of the peritro- hic membrane possibly serves a useful purpose in preventing the regurgita- ion of the gut contents into the ampullae.

The tubules are arranged in an arc round the ampullae; those attached ntero-laterally form a bundle passing to the anterior parts of the body and hose attached postero-laterally to the posterior parts of the body (fig. 1, D). his condition is similar to that described in *Melanoplus* (Stuart, 1935), and s also similar to four species of Acridiidae illustrated by Bordas (1898, pl. V, ig. 3; pl. VI, figs. 1, 2, 3.). Six tubules, one from each of the 6 groups in the lorso-lateral, lateral, and ventro-lateral positions, pass directly backwards (as n fig. 1, A) and form a series of coils, in two layers, on the rectum. These 6 ubules are the primaries, the first-formed tubules of the embryo, and have onstant positions in all specimens. The secondary tubules also make attach- ments to various organs of the body, but with what degree of consistency is unknown; some indeed occasionally enter the cavity of the heart.

The individual tubules are similar to one another except for slight differ- ence of size; each is a simple tube about 4 cm long and 50 to 100 μ wide, overed by a thin mesodermal layer of 'peritoneal cells' (Stuart in *Melanoplus*, 935), and supplied with a trachea running spirally along its length. Each tubule consists of a short upper segment 1·5 to 2·0 mm in length (fig. 1, E) and a long lower segment, much as in *Blatta* (Henson, 1944). The upper segment is clear and colourless, the lower segment relatively opaque, yellowish, and frequently containing crystals. Histologically, the two regions differ slightly, the cells and nuclei of the upper segment being smaller than those of the lower. Both show a striated border and 5 rows of cells in cross-section (fig. 1, H, J). Histochemical techniques, as used by Chauvin (1941), do not seem to be applicable in the present study.

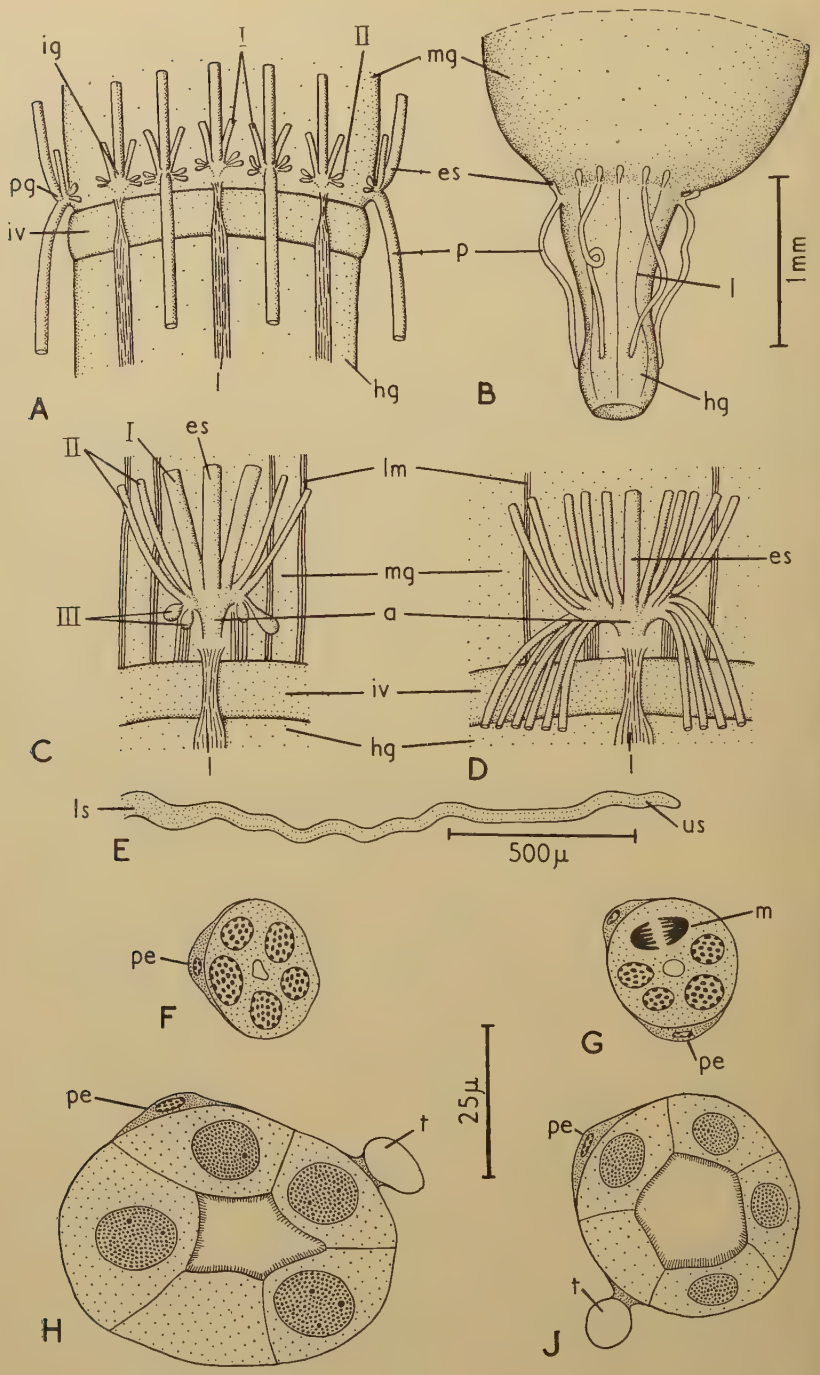

FIG. 1 (see opposite for description).

Embryo. The arrangement of tubules in the embryo is much simpler than the adult, 6 of the groups consisting of 2 tubules and 6 of only 1. These may be called 'primary' groups and 'intercalary' groups respectively (compare fig. 1, A, B). The primary tubules, emerging from the gut in the dorso-lateral, lateral, and ventro-lateral positions, pass backwards and become attached to the future rectal region of the hind-gut (figs. 1, B; 2, C), eventually to adopt the form described for the adult. The 12 embryonal secondaries, on the other hand, all pass along the mid-gut to anterior parts of the body. At this early stage the ampulla is little developed, being represented only by a small dilatation of the wall of the hind-gut (fig. 2, B). As in the adult, the tubules are composed of 5 rows of cells (fig. 1, F) but upper and lower segments are not distinguishable. It seems probable that this last differentiation is not achieved until functioning begins after hatching.

The Development of the Tubules

The primary tubules. In the 5- to 7-day embryo (at 25° C) 6 tubule-buds appear in the embryonic zone at the anterior end of the 'proctodaeum' just posterior to the so-called proctodaeal membrane (fig. 2, A). This zone has been shown to be homologous with the posterior imaginal ring of the higher Holometabola and is better known as the posterior interstitial ring (Henson, 1944). The buds first appear as groups of more actively dividing cells, and rapidly elongate to produce small tubules thickly covered with mesoderm (fig. 2, A). They continue to elongate posteriorly, the mesodermal covering gradually becoming thinner until it is only represented by a squamous epithelium (fig. 1, F).

Thus the primary tubules arise from what appears to be the proctodaeum. If, however, an adult locust is examined it is seen that the primary tubules are inserted on the mid-gut and separated from the hind-gut by mid-gut tissue. Similar conditions with their implied contradictions were encountered by Henson in *Blatta* (1944) and were resolved by a re-interpretation of insect gastrulation (Henson, 1946*a*). It was shown that this embryonic zone, i.e. the posterior interstitial ring, at the inner end of the proctodaeum is homologous with the anal half of the blastopore of *Peripatus*. Tissue produced posteriorly from this zone constitutes hind-gut and is ectodermal whilst tissue produced anteriorly constitutes mid-gut and Malpighian tubules, and is endodermal. This latter region lies between the mesenteron proper and the hind-gut and was named the met-enteron. Its anterior boundary is thus not a proctodaeal

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Fig. 1. A, dorsal view of alimentary canal in an early second instar. B, ditto, in 9-day embryo. C, dorsal intercalary group in an early third instar. D, ditto, in an adult. E, distal region of an adult tubule. F, transverse section of a primary tubule in an embryo. G, ditto, embryonal secondary in an embryo. H, ditto, lower segment of adult tubule. J, ditto, upper segment of same tubule. *a*, ampulla; *es*, embryonal secondary; *hg*, hind-gut; *ig*, intercalary group; *iv*, ileo-ventricular sphincter; *l*, longitudinal muscle of hind-gut; *lm*, longitudinal muscle of mid-gut; *ls*, lower segment; *m*, mitosis; *mg*, mid-gut; *p*, primary; *pe*, 'peritoneal cell'; *pg*, primary group; *t*, trachea; *us*, upper segment; I, first instar secondaries; II, second instar secondaries; III, third instar secondaries.

FIG. 2. A, the origin of a primary tubule in a 6-day embryo. B, the origin of primary and embryonal secondary tubules in a 14-day embryo. C, transverse section of future rectal region of the hind-gut in a 9-day embryo. *c*, chitinous intima of hind-gut; *es*, embryonal secondary; *f*, fold from posterior margin of primary to met-ental membrane; *hg*, hind-gut; *ia*, dilatation of hind-gut forming initial stage of the ampulla; *m*, mitosis; *me*, mesoderm; *mg*, mid-gut; *p*, primary; *pr*, 'proctodaeal' or met-enteric membrane.

embrane but a met-enteric membrane separating two constituent parts of e mid-gut, not mid-gut and hind-gut as previously assumed. Malpighian bules are thus met-enteric outgrowths of endodermal derivation and not todermal outgrowths of the proctodaeum.

Tubule anatomy in *Schistocerca* corresponds precisely with this interpretaon. Fig. 2, A shows the inner end of the proctodaeum (*hg*), the met-enteric embrane (*pr*), and the origin of the primary tubule. Comparison with a fifth star (fig. 3), which shows the posterior interstitial ring more clearly than the dult, shows that the tubule arises on the anterior side of the true innermost nd of the proctodaeum and is therefore endodermal.

From the earliest stages of development the tubules have a wall 1 cell ick and 5 cells in cross-section. Observations suggesting other than 5 cells rise from variations in the plane and thickness of the sections. The lumen is mall, there is no striated border, and the constituent cells are small with arkly staining cytoplasm and relatively large nuclei (fig. 1, F). Almost all itoses are in the longitudinal direction (fig. 2, A, B) and it is clear that elongaon is related to such divisions. Only rarely have transverse divisions (fig. 1, G) een seen and a perpendicular one never. Thus the cellular constitution of the bule at this stage of development is associated with, but not necessarily ependent upon, the control of the direction of cell division. As a result of is posterior elongation all the tubules pass backwards and become attached the future rectal region of the hind-gut (fig. 2, C). Mitoses now cease and rther elongation and differentiation are brought about by cell enlargement nd a little rearrangement.

The time at which mitoses cease corresponds to the beginning of the forhation of the embryonal secondary tubules; in fact no mitosis has ever been en in a primary once the secondaries have been initiated. As will be seen ter, a similar correspondence is met with in nymphal stages. During the emainder of embryonal life the tubules increase in size slightly and begin to rm the coils on the rectum. It will be remembered that the tubules are alady attached at both ends and hence coiling must depend mainly on two ctors: the differential elongation of the hind-gut and the tubules, and the ature of the arrangement and enlargement of the constituent cells of the ubules. A single layer of coils is formed during early stages and a second yer on top of this during nymphal stages. The form of the coils is much the me in all specimens, indicating that a specific morphogenetic process is volved.

Soon after hatching, the tubules, although still short of their full length for ne first instar, begin to show signs of functioning. This is indicated by the ct that upper and lower segments are now recognizable, the former clear and olourless and the latter yellowish and relatively opaque, as in the adult. At this tage the upper segment has already attained its full adult length and is about ne-third of the total length of the whole tubule. This implies that its differenation is already almost complete, and during the remainder of its development here is only an increase in diameter and a progressive decrease in its length

FIG. 3. Longitudinal section of the ampulla in a fifth instar. A, ampulla; *c*, chitinous intima
hind-gut; *ct*, connective tissue; *hg*, hind-gut; *hgc*, hind-gut cells of ampulla; *me*, mesoderm
met, met-enteron; *mg*, mid-gut; *mgc*, mid-gut cells of ampulla; *miv*, muscle of ileo-ventr
cular sphincter; *mu*, muscle; *pir*, posterior interstitial ring; *pt*, peritrophic membrane; *t*
tubule.

proportional to that of the lower segment. In the latter the lumen is somewhat larger (fig. 4, C) and there is a striated border; in fact all the characteristics of a fully functioning tubule may be observed.

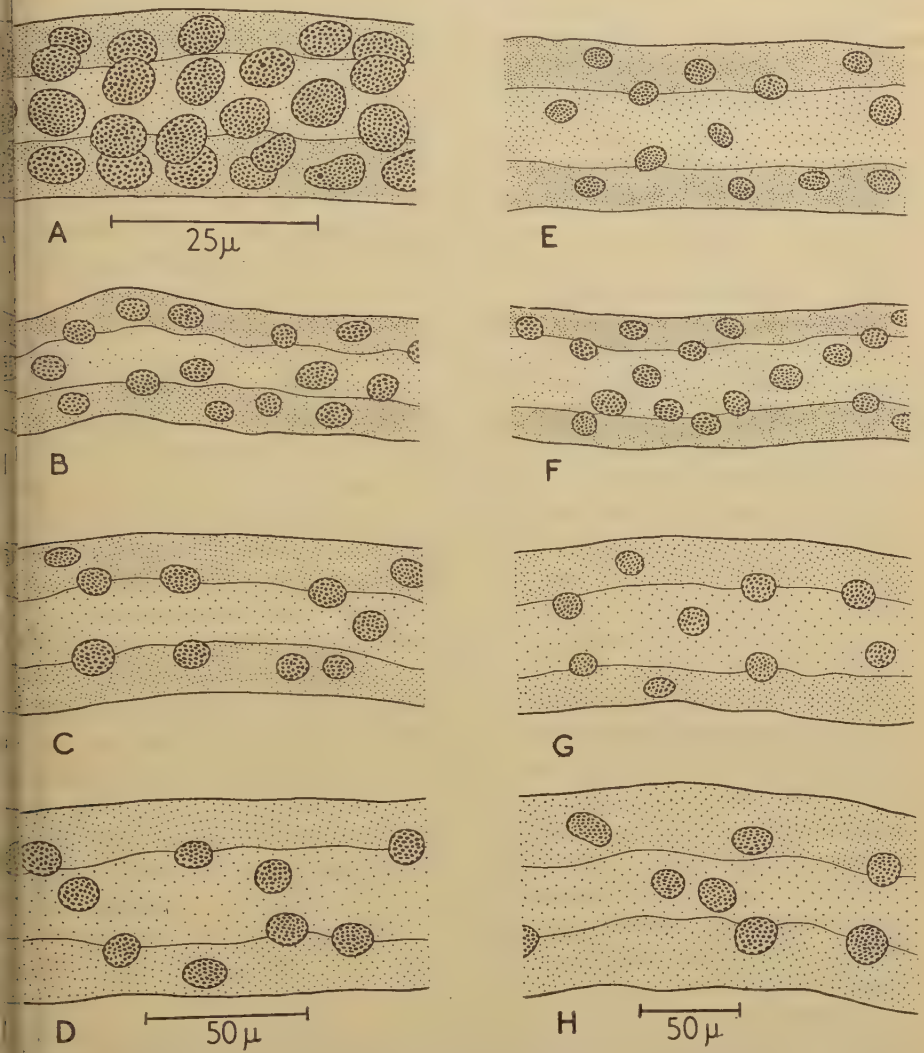

FIG. 4. Surface view of the primary tubules (from life). A, early embryo. B, late embryo. C–G, 1st–5th nymphal instars. H, imago.

Throughout the remainder of development the tubule continues to increase in size. This increase takes place not continuously but in a 'stepped' fashion. During each nymphal stadium the increase in size is gradual, but at each ecdysis the tubules become smaller again without actually becoming as small as at the previous ecdysis. This is probably associated with loss of water at moulting. A comparison of fig. 4, E (drawn many days after ecdysis) and

fig. 4, F (drawn a few hours after ecdysis) illustrates this phenomenon. Hence the gradual growth of the tubules is only observed if they are examined at comparable times in each instar (fig. 4). It will also be seen from the figure that the lumen progressively enlarges, the cytoplasm becomes less dense, and the nuclei increase in size as the adult state is attained. There is some variation in the size of nuclei in any given tubule and the increase in size is a gradual process, there being no sudden significant increase as was observed in *Forficula* (Henson, 1946*b*). Similarly smaller cells are associated with smaller nuclei in the upper segment (compare fig. 1, H, J). Throughout this later stage of development the wall of the tubule remains 1 cell thick with 5 rows of cells in cross-section, precisely as in the earlier mitotic stage (fig. 1, H, J). The cells, as shown by the position of the nuclei (fig. 4), tend to run spirally round the tubule, as also does the trachea.

The primary tubules of *Schistocerca* are thus composed of 5 rows of spirally arranged cells. Development consists of a mitotic phase followed by a phase of cell enlargement. The arrangement of the cells is attained early in the mitotic phase and is not seriously disturbed thereafter. It may be noted here that this pattern of development is different from that of other forms which have been investigated (*Pieris*, *Forficula*: Henson, 1937, 1946*b*).

The embryonal secondary tubules. In the 9- to 10-day embryo 12 more tubule-buds appear, 6 immediately anterior to each of the 6 primary tubules and 6 in the intervening or intercalary positions, i.e. opposite the future longitudinal muscle-bands of the hind-gut (fig. 1, B). These 12 tubules, the embryonal secondaries, all pass forwards to anterior parts of the body in contrast to the primaries, which pass backwards to the rectum. As in the case of the primaries they arise in the posterior interstitial ring from groups of actively dividing cells which then elongate. From the very beginning these too have a wall 1 cell thick and show 5 rows of cells in cross-section. The mitotic phase of development, which establishes this latter arrangement, ceases before the formation of the first instar nymphal secondaries; just as the mitotic phase of the primaries ceased before the initiation of the embryonal secondaries. Further increase in size is brought about by cell enlargement with perhaps a little rearrangement, but they never become quite as large as the primaries even by the imaginal stage.

Immediately after their formation the lumen of these tubules, like those of the primaries, open freely into the gut. In the case of the 6 primary groups (fig. 1, A), a forwardly directed membrane now develops from the posterior margin of the primary tubules and extends inwards to the met-enteric membrane (fig. 2, B). This membrane disappears at the time of hatching along with the met-enteric membrane and has no connexion with the fold along the posterior border of the adult ampulla.

Thus in the late embryonal stages of the locust there are 18 tubules arranged at 12 points round the gut, marking the future positions of the ampullae.

The nymphal secondary tubules and the ampullae. In each of the 5 nymphal instars new secondary tubules arise in the region of each of the pre-existing 12

groups. They elongate and differentiate, and at the same time contribute to the formation of the ampullae. All new tubules are added postero-laterally on each side of the one or two existing tubules constituting one or other of these groups: no new groups are formed.

In the first instar two lateral tubules are normally added to each group, one on each side of the existing embryonal secondaries along the line of the posterior interstitial ring between mid-gut and hind-gut (fig. 1, A, C, I). In the following instars further tubules are added, once more postero-laterally (fig. 1, A, C, II, III), until the typical arc-shaped arrangement of the adult is established (fig. 1, D). Each tubule arises as a group of actively dividing cells in the interstitial ring and its development is divisible into a mitotic phase and a cell enlargement phase as described for the embryonal tubules. The mitotic phase once again ceases before the initiation of the tubules of the next instar. They show exactly the same cellular construction as the earlier tubules but remain rather smaller, even when adult.

The nymphal secondary tubules produced in each instar always occupy definite positions in the body cavity of the insect. Those formed in the first and second instars all pass forwards, those formed in the fourth and fifth instars all pass backwards, whilst of those formed in the third instar some pass backwards and some forwards. This arrangement and the extent of development of the ampulla are thus interdependent and explain the arrangement of the adult tubules in two bundles, one running backwards and one forwards (fig. 1, D). Other Orthoptera have a similar arrangement of tubules (Bordas, 1898; Stuart, 1935) and probably similar modes of development.

The formation of the ampullae is the ultimate result of the arc-shaped arrangement of the tubules around it. This in its turn depends upon changes in the form of the posterior interstitial ring; originally annular, this becomes undulating by passing forwards at the level of each tubule group. The U-shaped form of the group of tubules is thus the result of the U-shaped form of the posterior interstitial ring at this level (figs. 1, D; 5, E). The ampulla first appears in the late embryo, a few days before eclosion, as a small dilatation of the hind-gut resulting from the forward bending of the posterior interstitial ring (figs. 2, B; 5, A, B, C). Its increase in size results from the successive addition of tubules round the rim of this zone, followed by the production of mid-gut cells immediately behind each tubule. This further production of mid-gut cells seems to be continued through later stages because more mid-gut tissue is present behind the older tubules than behind those recently formed (fig. 5, D). This is to say that much of the outer wall of the ampulla is now mid-gut, situated posteriorly to the Malpighian tubules. The rest of the posterior outer wall, i.e. that posterior to the interstitial ring, is still hind-gut. Mitotic figures have been seen on this hind-gut side and show that the ring is still active in the production of new hind-gut cells posteriorly (figs. 3; 5, D). The tubules, therefore, in their fully developed condition are definitely inserted on the mid-gut and not at the mid-gut/hind-gut junction as so often asserted. The original forward bending of the interstitial ring in the late embryo is

probably due to the formation of new hind-gut cells at this point. The evidence for this is rather slender, but mitotic figures have been seen on this side.

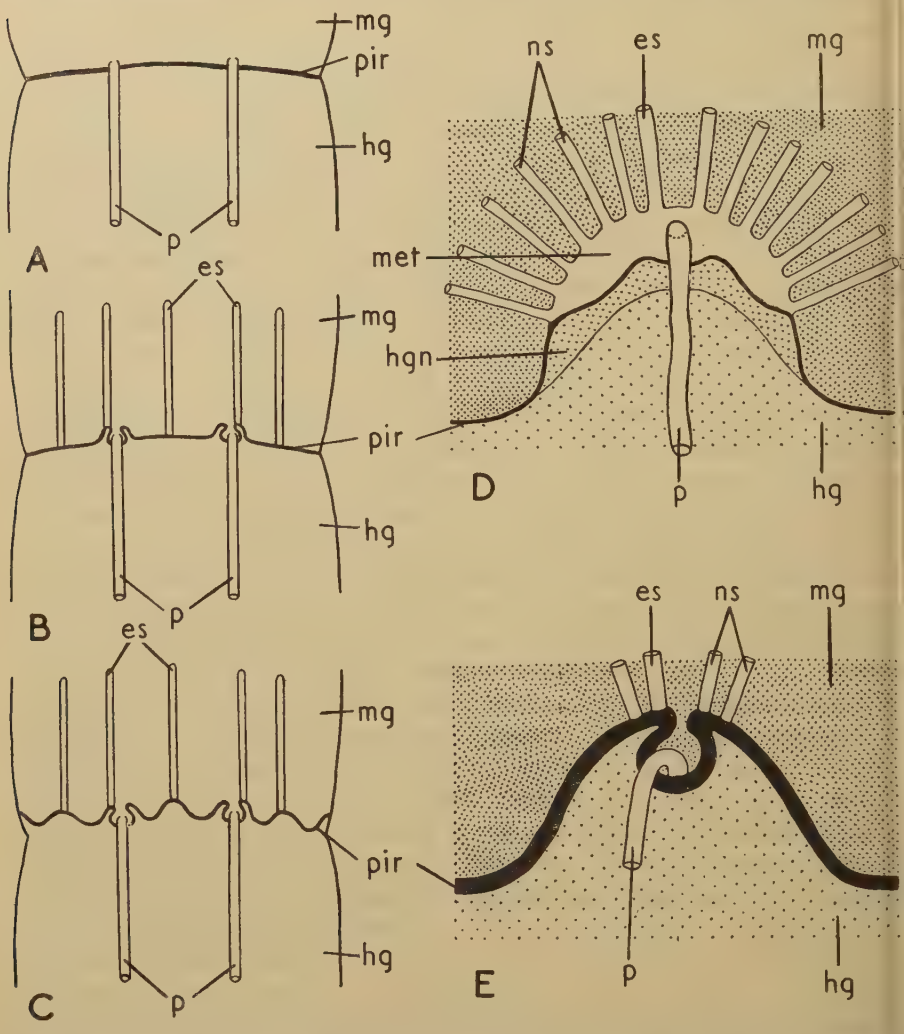

FIG. 5. The form of the posterior interstitial ring (theoretical). A, dorsal view of alimentary canal at mid-gut/hind-gut junction in early embryo. B, ditto, in late embryo. C, ditto, just before eclosion. D, dorsal view of a tubule group in a third instar nymph. E, ditto, in first instar. *es*, embryonal secondaries; *hg*, hind-gut; *hgn*, new hind-gut; *met*, met-enteron; *mg*, mid-gut; *ns*, nymphal secondaries; *p*, primaries; *pir*, posterior interstitial ring.

The increase in the number of tubules. As already mentioned, the Malpighian tubules of the locust arise in 7 successive cycles throughout the pre-adult life, thus increasing the number of tubules from 6 to about 250. The first generation of 6 tubules arises in the early embryo as small buds which rapidly elongate before any sign of the second generation is apparent. In turn the latter, the

embryonal secondaries, also elongate so that 18 well-developed tubules are present at the time of hatching. The first instar secondaries, consisting of two tubules at each group position, do not appear, even as buds, until 1 or 2 days after hatching. These two arise not quite simultaneously but one after the other and become well developed by the end of the instar. Similarly, at the beginning of the other instars, the buds in each group arise successively over a short period of a few days and in turn become elongated before the next ecdysis. In the second to fourth instars about 6 to 8 tubules are formed in each group as against 1 or 2 in the first and fifth. Since their production continues to be successive, the period of time over which they are formed must be appreciably longer. The development of the secondary tubules in the nymphal instars is thus very similar to the development of the embryonal tubules. Mitosis sets in at some predetermined position within a group, produces a bud, and continues during the period of elongation right up to the end of the stadium.

In the first to the fourth instars one or two of the last-formed buds may cease to develop and remain dormant until the following instar. It is as though development were arrested soon after elongation had set in. After the next ecdysis, however, development continues as if they were the first-formed tubules of this new instar. Tubules of intermediate length are never found in a condition of dormancy; at the end of every instar the tubules are either short buds or more or less fully elongated. Thus the developmental sequence of any one tubule can be arrested up to a certain point and the mitotic phase of development may be divided into two periods: an earlier one during which it is capable of being arrested and a later one during which it is not.

The cycle of development of any one tubule thus consists of a period of initiation followed by a period of mitosis, during which elongation takes place, and this again by a period of cell enlargement which continues to the adult state. The period of initiation is restricted to a few days at the beginning of the stadium; the mitotic phase continues throughout the same stadium and is brought to a close just before ecdysis sets in; cell enlargement proceeds continuously throughout the rest of the life of the tubule independently of instars and ecdysis.

The restriction of the period of initiation to the first few days of the stadium seems to be the result of some factor operating in the insect as a whole, presumably a humoral influence. The occurrence of dormancy shows that the period of mitosis can be arrested at a relatively early stage as well as at the end of the stadium. Since only late-formed tubules are so affected this too can be interpreted in terms of a humoral influence, but such interpretation is not simple, since once the tubule gets beyond a certain point its continued elongation and mitosis are not disturbed. The situation is much the same as in the analysis of the growth of the antennal joints of *Forficula* (Henson, 1947), where the character of the growth cycle is an attribute of the organ itself and the animal as a whole exerts its influence merely in determining the rate at which this cycle shall proceed.

Although the number of tubules added in each instar is subject to variation, their points of origin relative to one another are absolutely constant. The 6 primary tubules arise first and are followed by the 12 embryonal secondaries. Of the latter, 6 arise alongside the primaries and are apparently situated immediately in front of them, and 6 occupy the intercalary positions, thus establishing the 12 groups (figs. 1, A; 5, B). The anterior position of the 6 on the primaries is anomalous and can only be explained by assuming a somewhat involved bending of the posterior interstitial ring round the anterior side of the primary (see below). In the first instar 2 tubules are normally added, 1 on each side of the existing embryonal secondary, along the line of the posterior interstitial ring. Hence there are 2 tubules on one side of the primary and 1 on the other. In the second instar a variable number of tubules is added to each group, also along the line of the posterior interstitial ring. Four to six is the usual number and they are always arranged symmetrically, in a numerical sense, along each side of the already established group. This is to say that if 6 are formed, there are 3 on the left and 3 on the right. The continuance of additions left and right means that the groups are balanced as regards number, never showing an asymmetry greater than one. In the last case the first-developed tubule of the next instar is on the side with the fewer number and restores the symmetry. Even in the adult the numerical asymmetry is never greater than one, thus revealing the absolute constancy of this developmental sequence (figs. 1, D; 5, D, E).

The anomalous anterior position of the embryonal secondaries may now be explained as follows. The alternating series, of which they are the first, is lateral to the primary and therefore they themselves must be morphologically lateral. In fact many instances have been observed in which they do not occupy a strictly median position. It would seem that the line of the posterior interstitial ring is bent forwards round each side of the insertion of the primary as shown in fig. 5.

The actual number of tubules formed in the embryo and in each instar is shown in table 1. No variation has ever been encountered in the number present at hatching; all specimens have 18 tubules. After this, however, a good deal of variation occurs. The maximum increase takes place in the third instar and there is a surprisingly small increase in the fifth. In this last stage only one tubule may be added but no case in which no addition had taken place was ever noticed. The occurrence of dormancy renders an analysis of tubule numbers rather less significant than might appear at first sight, since buds properly belonging to one instar may not develop until the subsequent one. Dormancy, however, does not explain the whole of the observed variation.

Discussion

The primitive number of Malpighian tubules in insects has been discussed on many occasions in the past. Wheeler (1893) surveyed all existing literature and concluded that the primitive number was 6. He pointed out that no known embryo had more than 6 tubules and that forms with fewer than 6 could

eadily be derived from the primitive type. Henson (1944) suggested that the primitive condition was 6 primary tubules with numerous secondaries, and hat the higher Pterygota could be derived from the more primitive types by uppression of secondary tubules. Until the present, however, no such primiive type with 6 primaries and numerous secondaries has been fully described. t will be appreciated that the desert locust provides this hitherto hypothetical orm.

TABLE 1

Generation	*Stage*	*Number examined*	*Number of tubules*		*Increase in no. of tubules*	
			Range	*Mean*	*Range*	*Mean*
1	1st phase embryo 4-day	15	0	0		
	7-day	11	6*	6	6*	6
2	2nd phase embryo 8-day	16	6*	6		
	10-day	13	18*	18	12*	12
3	1st instar early	53	18*	18		
	late	17	34–50	43	16–32	25
4	2nd instar early	8	34–50	43		
	late	9	83–124	94	36–74	51
5	3rd instar early	6	83–124	94		
	late	12	151–221	182	68–91	88
6	4th instar early	5	151–221	182		
	late	7	217–85	245	50–75	63
7	5th instar early	8	217–85	245		
	late	10	220–94	251	1–10	6
	Imago	14	220–94	251		

* No variation observed.

The anatomy of the tubules of *Schistocerca gregaria* is essentially similar o that of many other Orthoptera, which have been described as having the ubules arranged in a number of groups opening into the alimentary canal at he junction of mid-gut and hind-gut (Bordas, 1898). Nevertheless, the details of group arrangements are insufficiently known for any worth-while discussion. It seems that no one characteristic arrangement is peculiar to any one suborder, and so for the present no classificatory value can be assigned to tubule arrangement.

The cyclical nature of insect development has long been known, but only in recent years has the relationship of this form of development to development in general begun to be appreciated. Henson (1946*a*) suggested that metamorphosis and the developmental cycles associated with ecdysis are essentially similar to each other and to embryogenesis. Wigglesworth (summarized 1954) has further shown that each inter-ecdysial cycle is capable of modification and may take any one of a number of different courses, depending upon the humoral state of the insect. The results of the present investigation may be interpreted in accordance with the views of these authors. In the locust, as well

as in *Blatta* and *Forficula* (Henson, 1944, 1946*b*), the formation of Malpighian tubules is cyclical and is clearly one aspect of the inter-ecdysial developmental processes. In *Schistocerca* there is one generation of tubules in each nymphal instar and two in the embryo and hence the total number of developmental cycles is seven. The conception of two embryonal cycles in *Schistocerca* is supported by the existence of the associated 'intermediate moult' (in Uvarov, 1928) and by the activity of the prothoracic gland associated with a similar embryonal moult in *Locustana pardalina* (Jones, 1953).

The details of the development of individual tubules concern initiation, mitosis, and elongation, and differentiation as revealed by cell-enlargement and development of the striated border. It has been shown that all generations of tubules, both embryonal and nymphal, follow the same pattern, initiation and mitosis in one cycle (instar) followed by differentiation in subsequent cycles (instars). In other words, the pattern of development of a nymphal tubule is precisely the same as the pattern of an embryonal development and this conclusion strongly supports the suggestion that inter-ecdysial cycles and embryogenesis are one and the same thing.

The relationships between the development of Malpighian tubules and the interpretation of insect gastrulation have already been considered by Henson (1932, 1946*a*). Nothing in the present investigation is out of harmony with the views there expressed. It was shown that in many insects there is an annular ring of embryonic cells at the inner end of the proctodaeum, the posterior interstitial ring, homologous with the anal half of the Annulate blastopore. In *Schistocerca* this ring is active at 12 points round the gut and tubules are formed laterally along it. The details have already been described and it will be appreciated that the essential condition that Malpighian tubules remain on the anterior side of the interstitial ring is fulfilled. Furthermore, the formation of the ampulla is dependent upon the activity of the ring since it produces mid-gut cells anteriorly and hind-gut cells posteriorly. The mid-gut cells so produced correspond to the met-enteron (Henson, 1946*a*). It would appear that the production of new hind-gut cells in nymphal stages, although to be expected, has not hitherto been recorded.

The basic features of tubule growth and differentiation are the same in all types so far described. Three main processes are involved, cell division, cell arrangement, and cell enlargement, the latter continuing throughout life up to the imaginal stage.

In *Blatta* and *Schistocerca* the tubules begin as small buds, in which there are abundant mitoses. The cells are in 5 rows right from the beginning and the direction of mitotic divisions is controlled in such a way that this arrangement is never seriously disturbed. Elongation is gradual throughout the instar. In *Forficula* the initial tubule-bud also has 5 rows of cells and for most of each stadium the process of elongation continues as in *Blatta* and *Schistocerca*. However, during the last 2 or 3 days before ecdysis mitosis ceases and re-arrangement of the tubule-cells sets in, reducing the number of cell rows from 5 to 2 and rapidly trebling the length of the tubule. In *Pieris* there are of course

only the primary tubules; these arise in the embryo as large buds consisting of about 15 rows of cells. Mitotic figures do not seem to be orientated in any particular direction and, even more important, they cease before cell rearrangement sets in. The latter process is more rapid than in *Forficula*, the cells becoming arranged in progressively fewer rows until only 2 are present.

In *Blatta* and *Schistocerca*, therefore, elongation and mitosis take place simultaneously and are continued throughout the instar, finally giving a tubule composed of 5 rows of cells. In *Forficula* the same sequence of events gives the same results until a new process of rearrangement sets in. This only begins after mitosis has ceased and results in a tubule composed of 2 rows of cells. In *Pieris* the cell rearrangement begins relatively much earlier, but still only after mitosis has ceased, and again produces a tubule consisting of 2 rows of cells.

In the more primitive types, therefore, elongation and mitosis take place simultaneously and occupy most of each stadium, whilst there is very little cell rearrangement. In the more advanced types cell rearrangement becomes increasingly important and gives a far more rapid increase in length. In *Pieris* the basic process—of cell division restricted to certain planes which result in elongation—is eliminated. In the more primitive types, therefore, cell multiplication and tubule building are simultaneous, whereas in more advanced forms directionally unrestricted multiplication precedes a separate process of tubule elongation by cell enlargement. The two processes of multiplication and cell arrangement have different temporal relations and different degrees of morphogenetic importance in the different types.

My thanks are due to Professor E. A. Spaul for kindly advice and facilities for study, and also to Dr. H. Henson who supervised this work. The material was supplied by Dr. B. A. Kilby and this investigation was made possible by a D.S.I.R. research grant.

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Muscle Receptor Organs in Grasshoppers and Locusts (Orthoptera, Acrididae)

By E. H. SLIFER AND L. H. FINLAYSON

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With one plate (fig. 2)

Summary

A pair of muscle receptor organs is present in each of the abdominal segments from the first to the tenth in the Acrididae. They are associated with the dorsal longitudinal muscles and each consists of a single, heavily encapsulated sensory neurone with numerous dendrites which end on a long, slender muscle-fibre which has its origin near the anterior end of the segment and is inserted on the anterior border of the segment next posterior to it.

PAIRED, segmentally arranged sensory structures which are associated with the dorsal longitudinal muscles of the thorax and abdomen have been described by Alexandrowicz (1934, 1951, 1952*a*, 1952*b*, 1954, 1956) for species of Crustacea belonging to several different orders. Alexandrowicz gave them the name of muscle receptor organs and suggested that they function as proprioceptors and are sensitive to alterations in the length of the body as it is extended or contracted. His suggestion concerning the function of these structures in Crustacea was confirmed by physiological experiments which were reported by Wiersma, Furshpan, and Florey in 1953. Florey and Florey (1955) made a detailed study of the anatomy of the muscle receptor organs of *Astacus*. In 1955 Finlayson and Lowenstein described similar structures in the larva, pupa, and adult of several species of silkworm moths. They examined the organs with the aid of an oscillograph and found that electrical impulses from the nerve increased in frequency when the organ was stretched and decreased again when the tension was released.

Muscle receptor organs have also been identified recently in the Acrididae and the present paper is concerned with a description of their major structural features.

Materials and Methods

The species of Acrididae with which most of this work was done were *Locusta migratoria migratorioides* (Reiche and Fairmaire), *Romalea microptera* (Beauvois), and *Schistocerca gregaria* (Forskål), while *Dissosteira carolina* (Linnaeus), *Melanoplus mexicanus mexicanus* (Saussure), and *Phoetaliotes nebrascensis* (Thomas) were examined in less detail. Whole mounts of the dorsal body-wall, together with the dorsal vessel, dorsal muscles, and the principal nerves which innervate the abdominal terga, were fixed in Bouin's

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fluid and treated very briefly with Mallory's triple connective tissue stain so that only the outer layers of thick structures were coloured. These preparations proved helpful in locating the muscle receptor organs during the earlier stages of this investigation. Other specimens were vitally stained either by immersion of dissected parts in 0·5% methylene blue in Ringer's solution or by injection of the dye into the whole animal. Fixation was in ice-cold 8% ammonium molybdate to each 10 ml of which were added 5 drops of 2% osmium tetroxide. The material was left in the fixative in the refrigerator for at least 12 hours, then washed, dehydrated very rapidly with the higher alcohols and dioxan, cleared in xylene or toluene, and mounted in HSR synthetic resin. Sections were made of material which had been fixed in Bouin's fluid and these were stained with Mallory's triple connective tissue stain, picro-fuchsin, or Heidenhain's iron haematoxylin.

Results and Discussion

In the Acrididae a pair of muscle receptor organs is present in each of the abdominal segments from the first to the tenth. One member of a pair is located on each side of the dorsal mid-line. In the first to the eighth abdominal segments the receptor organ is attached to the medial edge of one of the longitudinal muscle bands which have their origin near the anterior edge of each segment and are inserted on the anterior border of the next segment behind. The particular dorsal longitudinal muscle band to which the organ is attached is usually the fourth, fifth, or sixth from the mid-line but there is some variation even within a single species. Since, however, the number of muscle bands is, itself, also subject to some variation, this is not surprising. In one exceptional case the muscle receptor organ on the left side of the fifth abdominal segment of a male *Romalea microptera* was found to be lying near the middle of a muscle band instead of at its edge. In the modified ninth and tenth segments at the posterior end of the abdomen the receptor organs are located near the centre of a muscle band. Each muscle receptor organ consists of a slender fibre and a single, large sensory neurone which is provided with a number of dendrites (figs. 1 and 2, A, C). The neurone, which is usually located somewhat posterior to the middle of the fibre, is surrounded by a thick capsule and a number of neurilemma cells are associated with it (fig. 2, B). Nothing corresponding to the 'ovoid sac filled with an unidentified substance which stains with some nuclear stains such as haematoxylin and celestin blue', which was described for the Lepidoptera by Finlayson and Lowenstein (1955), was found in the Acrididae. The axon of the sensory neurone passes anteriorly and laterally and then joins the ventral branch of the tergal abdominal nerve of the segment in which the receptor is located (figs. 1 and 2, A). The slender fibre with which the sensory neurone is associated is a modified muscle-fibre as Finlayson and Lowenstein found it to be in the silkworm moths. The striations of the fibre are distinct but less conspicuous than are those of the nearby muscle-fibres. Numerous, thick connective tissue-fibres are attached to this specialized muscle-fibre throughout its

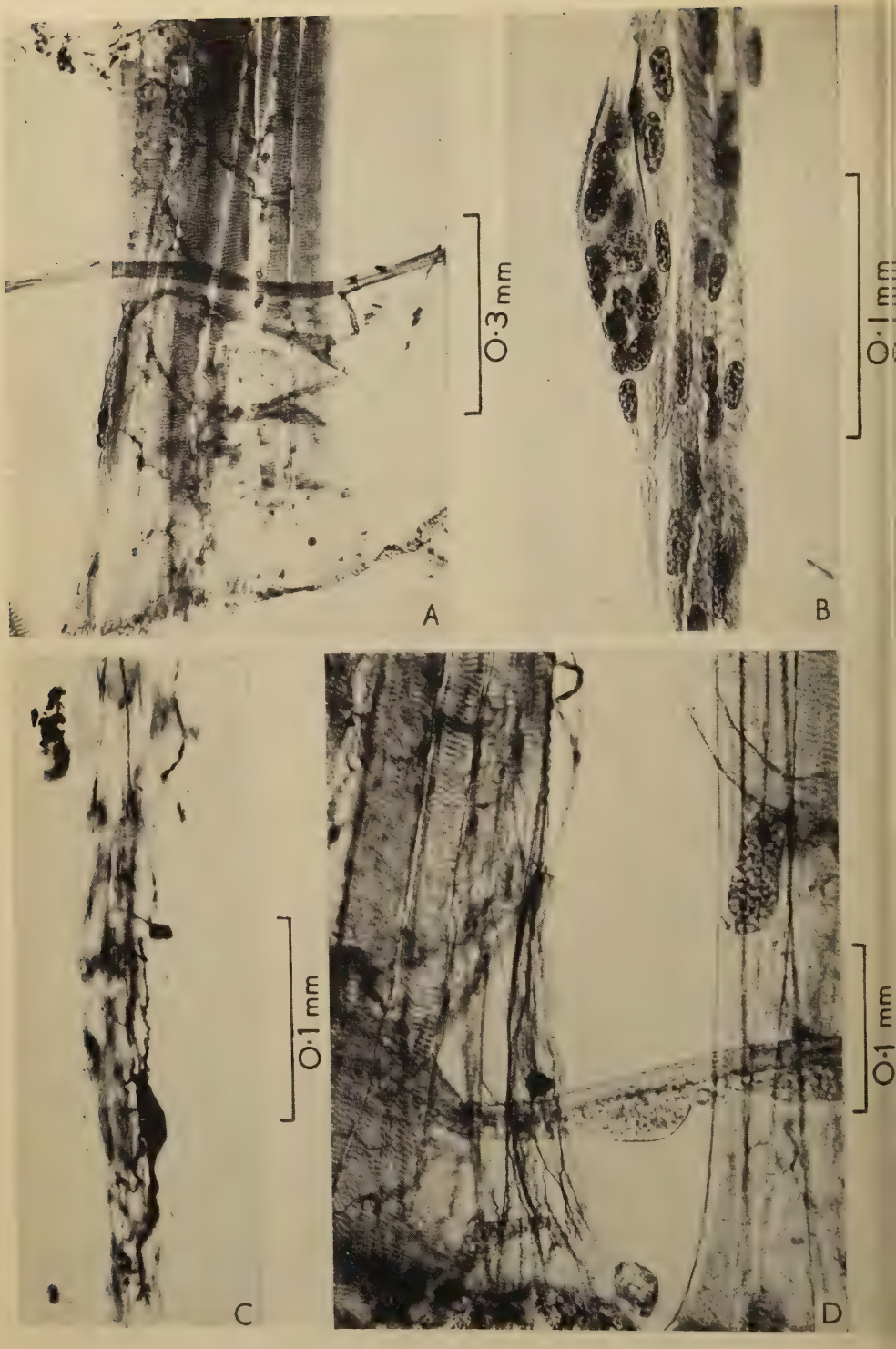

FIG. 2

E. H. SLIFER *and* L. H. FINLAYSON

ength, and in some preparations they stain heavily with methylene blue fig. 2, D). They are especially abundant close to and posterior to the neurone.

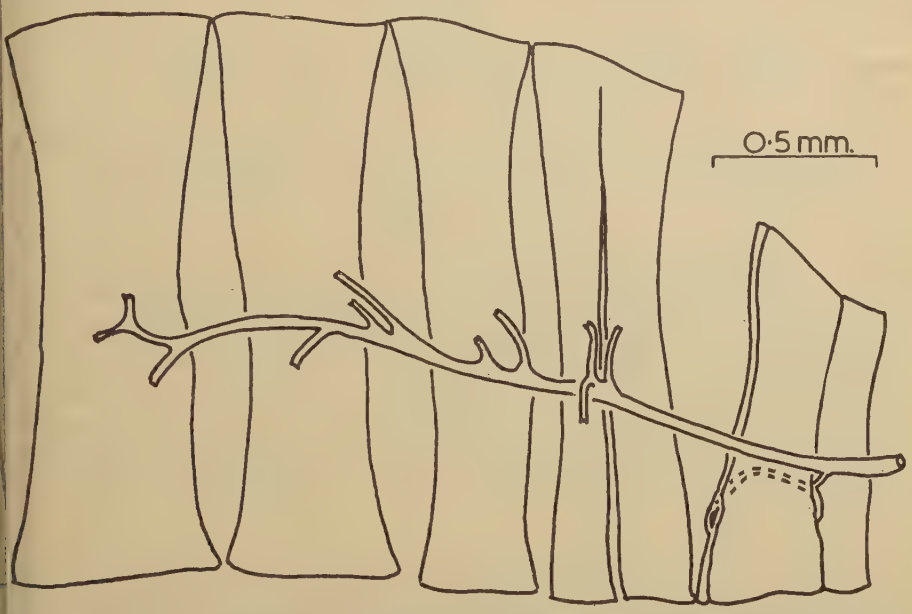

FIG. 1. Diagram showing relation of muscle receptor organ to dorsal longitudinal muscles of left side of second abdominal segment of a male adult *Locusta migratoria migratorioides*, as seen in ventral view. Mid-dorsal line at left. Slender muscle-fibre of receptor organ attached to medial edge of muscle band at extreme right. Encapsulated sensory neurone (darkened) posterior to middle of fibre. Nerve from receptor organ joins ventral branch of tergal abdominal nerve; other branches of tergal nerve, which provide a rich motor innervation for dorsal muscle bands, omitted except for short stubs.

Some of these connective tissue-fibres spread out over adjacent muscle-fibres and in this respect the receptor organ in the Acrididae differs from that of the Lepidoptera, where the connective fibres are confined to the modified

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FIG. 2 (plate). A, posterior two-thirds of whole mount of a dorsal longitudinal muscle band with muscle receptor organ attached to its left edge from left side of second abdominal segment of a male adult *Locusta migratoria migratorioides*, as seen in ventral view. Sensory nerve from darkly stained neurone of receptor organ joins ventral branch of tergal abdominal nerve near right margin of muscle band. Methylene blue preparation.

B, part of whole mount of muscle receptor organ showing large, spherical nucleus of encapsulated sensory neurone surrounded by the nuclei of a number of smaller (neurilemma?) cells which are associated with it. Adult male *Romalea microptera*. Picro-fuchsin.

C, part of whole mount of muscle receptor organ from right side of third abdominal segment of a male *Romalea microptera*, showing sensory neurone with axon and dendrites. Methylene blue. The straight fibres in the upper (anterior) part are connective tissue fibres. The axon disappears out of focus for part of its way and reappears on the extreme right at the top of the photograph.

D, part of whole mount of dorsal longitudinal abdominal muscle band from right side of eighth segment of adult male *Locusta migratoria migratorioides* to show wavy, branched connective tissue fibres associated with muscle receptor organ. Compare with smooth edge of ordinary muscle band at right. Methylene blue.

muscle-strand. It seems probable that the muscle receptor organ of the Acrididae, since it is still intimately associated with the ordinary muscle adjacent to it, represents a more primitive condition than does that of the Lepidoptera, where separation from the dorsal longitudinal muscles is complete. Finlayson and Lowenstein found muscle receptor organs in the thorax as well as the abdomen of caterpillars but were unable to find receptor organs in the thorax of the pupa and the adult. It is possible that muscle receptor organs are present in the thorax of the Acrididae but as yet none have been found. The heavy and complex musculature of this region makes it difficult to locate such structures if they do occur, and a more intensive search may still disclose them.

As yet no satisfactory physiological data concerning the muscle receptor organs in the Acrididae have been obtained, but it seems probable that the receptors will eventually be found to respond in much the same way as do the very similar structures which are present in the abdomen of the Lepidoptera. Since the abdomen of the female grasshopper or locust is greatly elongated when filled with mature eggs and stretched even more drastically during the deposition of the eggs in the soil, it seems likely that receptors which are sensitive to muscular extension would have an especially important role to play at such times.

Note.—Dr. J. S. Alexandrowicz of the Marine Biological Laboratory at Plymouth was consulted during the early stages of the present investigation, and he very kindly made a series of methylene blue preparations of the stick insect, *Carausius morosus*, and sent them to the senior author. In this species muscle receptor organs are present not only in the abdomen but in the mesothorax and metathorax as well. (The prothorax was not present in any of these preparations, so the receptors may also occur there.) The muscle receptor organs in *Carausius* are similar to those described in this paper so far as their general features are concerned.

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Improvements in the Sudan Black Technique

By JOHN R. BAKER

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SUMMARY

The author's Sudan black technique has been improved by changes in fixation. One may fix as for the acid haematein test, or else in a modified Flemming's fluid. The former fixation allows very strong colouring by Sudan black, while the latter gives particularly faithful preservation of the form of the cells and their lipid inclusions.

DURING the past few years I have sought to improve my Sudan black technique (Baker, 1949), which is a general method for revealing lipids of all kinds. I have tried to make the test more sensitive. Many cytoplasmic globules contain but do not consist of lipid. Probably they are aqueous 'vacuoles' with lipid dispersed in them. Certain lipids, especially cerebrosides and phospholipids, are capable of such dispersion, and the vacuole containing them may be of quite low refractive index. Sudan black will only reveal the lipid content of such globules if special precautions are taken to prevent the escape of the lipid. Other lipid globules are so small that they may be overlooked unless the Sudan black acts very vigorously.

I have also sought to improve the fixation. The form of the globules was well preserved by the old method, but nevertheless there was room for improvement, and the cell as a whole was often somewhat distorted.

The two methods of fixation given here replace the earlier method.

Acid haematein (*AH*) *fixation.* I have found no method of fixation that allows stronger subsequent colouring with Sudan black than the method used in the acid haematein test for phospholipids (Baker, 1946). This is probably due to the presence of calcium ions in the fixative. The whole process, including the postchroming, should be carried out exactly as though one were going to perform the AH test.

Lewitsky-saline (*LS*) *fixation.* This method is to be preferred to the other when special emphasis is placed on perfection of fixation. I know of no other fixative that gives, in gelatine sections, such faithful preservation of the form of cells and their lipid inclusions.

Lewitsky (1911) introduced Flemming's fluid without acetic acid as a fixative for mitochondria. It is excellent for cytoplasmic inclusions in general, but suffers from the defect of causing uneven shrinkage of delicate cells. I have found that the addition of 0·75 % of sodium chloride removes this defect. I call the modified fluid Lewitsky-saline or LS.

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The following formulae will be found convenient in practice and will also show at a glance the relationship between LS and Flemming's strong fluid:

	Lewitsky-saline	*Flemming* (1882) *with full acetic acid*
	(*ml*)	(*ml*)
distilled water	1·0	0·8
chromium trioxide, 5 % aq. . . .	0·3	0·3
sodium chloride, 5 % aq.	0·3	..
osmium tetroxide, 2 % aq. . . .	0·4	0·4
acetic acid, 20 % aq.	..	0·5
	2·0	2·0

The amount of fixative (2 ml) is ample for the minute pieces of tissue that are suitable in cytological studies, but LS is stable and a large volume can be made up if this is desired. (Flemming's fluid deteriorates slowly owing to the oxidation of the acetic by chromic acid: the method of preparation suggested here is a good one because there is no waste.)

Tissues should be fixed overnight in LS and washed for 5 h in running water or repeated changes.

Embedding. This is carried out as in the old Sudan black method. It is often a great convenience to have a single gelatine block that is suitable both for allowing the strongest possible colouring with Sudan black and also for use in the AH test for phospholipids. A word of warning about this is necessary... Formalum (Baker, 1949) is always to be recommended for hardening gelatine blocks *except* when they are to be used for the AH test. (The alum acts as a mordant for haematein and this spoils the test.) Formaldehyde solution without alum must therefore be used if the gelatine block is wanted for both purposes. If the block is wanted for the Sudan black technique only, formalum is preferable.

Colouring. I have found no reason to change the procedure when AH fixation is used. It is legitimate, however, to extend the period in the Sudan black solution to 10 min if necessary.

After LS fixation the sections must be bleached before colouring, for otherwise it is impossible to know whether the blackness of globules is due to osmium or Sudan black. Hydrogen peroxide solution is suitable. The exact concentration is a matter of no importance. One may mix 10 ml of a '20 vol.' solution of hydrogen peroxide with 90 ml of distilled water. The sections are bleached until they have lost all trace of osmium deposit and then washed for a few minutes in changes of distilled water. Colouring in Sudan black lasts $2\frac{1}{2}$ to 10 minutes. Staining in carmalum should be omitted, as it is very feeble and diffuse after LS fixation.

I thank Miss B. M. Jordan for much practical help, as usual. Dr. J. T.-Y. Chou and Dr. B.-P. Clayton have kindly tried the methods and given me the benefit of their experience with them.

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